THE ROLE OF DIETARY FAT IN INCREASING EGG WEIGHT IN THE DOMESTIC HEN (*Gallus domesticus*)

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I declare that this thesis has been composed by myself.

The work presented has not been submitted to the

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Abstract

Dietary fat is thought to possess some property capable of increasing egg weight in laying chickens which is separate from its metabolisable energy and essential fatty acid content. This thesis describes a series of experiments performed to elucidate the mechanism by which egg weight is increased.

Both yolk and albumen weight increased in young hens (<30 weeks old) fed a diet containing 55 g/kg maize oil. Only albumen weight was affected in older birds (>48 weeks old). It is suggested that yolk achieves its theoretical maximum weight at a relatively early age and is not responsive to fat-feeding thereafter.

Aspects of triglyceride-rich lipoprotein metabolism were studied in relation to yolk deposition and albumen synthesis. Dietary maize oil, at 55 g/kg, caused a three-fold increase in lipoprotein lipase (LPL) activity in post-heparin plasma, though the VLDL level was not lowered. Oviducal tissues possessed considerable LPL activities and removed significant quantities of fatty acid presented as triglyceride in biologically labelled VLDL and portomicrons.

Plasma oestrogen levels rose in birds fed the 55 g/kg maize oil diet. Increased albumen protein, yolk protein and VLDL synthesis in response to oestrogen is discussed.

A large scale nutritional experiment, involving 1248 pullets, investigated the laying performance of hens fed isoenergetic diets containing maize oil, olive oil, coconut oil, fish oil and tallow at five levels of inclusion. Food intake and rate of egg production were similar on all dietary treatments. Response curves were fitted to describe the change in egg weight. A high correlation existed between plasma oestrogen and egg weight on the different dietary treatments. Possible means by which dietary fats could influence plasma oestrogen levels are discussed.

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I. GENERAL INTRODUCTION

The modern egg industry is one of the most efficient means of producing animal protein and, not surprisingly, has grown to an enormous size worldwide. The British egg industry alone produces over 40 million eggs daily, with an annual farm gate value of £506M (Anon, 1988). Improving the efficiency of egg production is an active area for many scientific disciplines. An attempt to improve the economic efficiency of the egg industry necessitates an examination of the relative production costs involved. The major cost in egg production is feedstuffs (77% of total costs) and subsequently much research is directed towards improving the efficiency of food conversion.

A second important consideration is that eggs are sold in structured markets according to defined weight increments and, hence, egg weight (or size) is an important factor determining the value of the product. In Britain, the size categories in which eggs are sold are at 5 g intervals with large financial incentives to produce eggs in the heavier size categories (Table 1.1).

The prospects for improving the efficiency of nutrient utilisation in the laying hen was reviewed by Morris (1972) who suggested that improvements might come from three possible directions. Firstly, the hen's efficiency may be enhanced by selective breeding. Secondly, manipulation of the hen's environment might improve efficiency. Thirdly, dietary manipulation may allow the feed conversion efficiency to be increased. Egg size is greatly influenced by genotype and selection of birds with light body weight, early point of lay with good egg weight, and higher egg yield has resulted in the efficient modern strains. Such selection has led to a dramatic increase in the

Table 1.1: The weights and prices of the egg size categories sold in the British market (Anon., 1988)

Size	1	2	3	4	5
Weight (g)	+70	65–70	60–65	55–60	50–60
Price (p/dozen)	75–100	68–93	64–86	60–80	52–72

annual yield of saleable eggs per hen from 185 in 1962 to 262 in 1987 (Anon, 1988). Manipulation of the hen's environment by adopting new husbandry practices has also been suggested to improve egg weight and the efficiency of egg production. It is generally well understood that an increase in environmental temperature will lead to a decline in food intake and an increase in the efficiency of nutrient utilisation provided that the thermal stress temperature is not reached (Morris, 1972). However, this husbandry policy has the disadvantage of decreased egg weight. Decreasing the day lengths during the pullet's developmental period delays sexual maturity and, subsequently, increases the weight of early eggs (Morris, 1985), but the nutrient efficiency is not improved. Lastly, Morris (1972) suggested that dietary manipulation could enhance egg production efficiency and egg size. Two of the major nutritional factors influencing egg size are protein and fat.

The optimum crude protein content in layer diets has been estimated to be 160 g/kg (Bolton and Blair, 1977). Protein levels slightly above or below this amount appear to have no effect upon egg size, as reported by Hochreich et al., 1958, (155-185 g/kg), Sadagopan et al., 1972, (120-200 g/kg), and Saxena et al., 1986 (150-190 g/kg). However, others have reported that higher protein levels (170 g/kg) increase egg weight relative to a low protein diet (130 g/kg) (Quisenberry and Bradley, 1962). It should be noted that in this last study, the low protein diet caused a reduction in body weight indicating that the hens were in a state of negative protein balance. It seems likely that the egg weight response is determined by the amino acid composition rather than by the absolute protein concentration of

the diet. March and Biely (1963a), for example, found basal diets supplemented with glycine depressed egg weight whilst those supplemented with lysine and methionine increased egg weight. In general, it can be assumed that, once the diet provides an adequate protein supply with an appropriate amino acid balance, especially with reference to methionine and lysine, manipulation of the protein content has a negligible effect upon egg weight.

An adequate protein supply is required to maintain an optimal rate of egg production though it is doubtful whether levels higher than 160 g/kg have any effect. Egg number was unaffected by protein level in the study of Saxena et al. (1986) and there was no effect of protein level above 170 g/kg in the study of Sadagopan et al. (1985). Morris (1985) suggested that the protein supply which optimises the rates of lay also optimises egg size and that there is little scope to adjust egg size by adjusting dietary protein supply.

One dietary factor that has been thought to increase both egg weight and food conversion efficiency is fat. This issue is the basis of the present study and the background to this phenomenon is reviewed in detail below.

II. DIETARY FAT AND EGG WEIGHT

Fat has been used in poultry diets since the 1940s as a concentrated energy source. An increasing amount of interest has been shown in fat recently and the supply from the UK animal feed-fat industry has almost quadrupled over the past decade (Lowe and Howells, 1984). Initial studies showed that the addition of fat to diets was well tolerated by a number of species and an improvement in the live-

weight gains was reported in rats (Pearson and Panzer, 1949), dogs (Siedler and Schweigert, 1952), turkeys (Touchburn and Naber, 1966) and chicks (Rand et al., 1958). The most consistent response noted with fat supplements was an improvement in the feed utilisation efficiency in terms of feed:gain ratios. The increases in feed conversion reported for liveweight gains are also present in egg production.

The first report of the effect of dietary fat upon egg weight was from a group of workers investigating why purified and semi-purified diets consistently produced smaller sized eggs compared to practical diets. In an attempt to demonstrate the presence of an "unidentified factor" in practical ingredients, Jensen et al. (1958) supplemented a purified diet with many components normally found in practical diets such as dried grass, barley, soybean oil meal, animal tallow and maize. Maize was found to cause the greatest increase in egg weight and also improved egg numbers and the hatchability of fertile eggs. The distribution of the "unidentified factor" in various fractions of maize such as maize bran, dried maize steep liquor, maize oil meal, maize gluten meal and maize oil was investigated by Jensen et al. (1958). They found that a dietary supplement of 3% (w/w) maize oil was as effective as 70% (w/w) ground maize in raising egg weight and concluded that the "unidentified factor" was fat-soluble. Subsequent work showed that neither autoclaving nor saponification affected the potency of the "unidentified factor" in maize oil, but oxidation inactivated the "factor" and depressed egg size, production

The possibility that the "unidentified factor" could have been one of the fat-soluble vitamins (A,D,E or F) or aided the availability of these vitamins can be discounted. Increases in egg weight have been reported using purified fatty acids and fatty acid esters (Quisenberry and Bradley,1962 and Schutze and Jensen,1963).Endogenous fat secretion within the alimentary tract would be sufficient for the absorption of fat-soluble vitamins which have not been reported to affect egg production when supplied at levels above those which alleviate symptons of deficiency.

Following the early studies by Jensen and co-workers that dietary fat is capable of increasing egg weight, much interest has evolved in this area. The literature contains a great number of reports of the effect of dietary fat on egg weight, ranging from acidulated soybean soapstock (Shutze et al., 1958) to arachis oil (Waring et al., 1968), to individual fatty acids (Creger and Couch, 1961). A selection of egg weight responses in some dietary fat types is given in Table 1.2 together with some of the practical details. The selection is not intended to be complete, but rather to be comprehensive with respect to the different types of responses that have been reported to a variety of dietary fats. The reasons for such varied reports of the effect of dietary fat upon egg size are numerous, but a major factor is the lack of uniformity in the experimental designs. For example, birds that are depleted of their linoleic acid body store generally respond to dietary fat supplementation with increased egg weight to a greater extent. Research has indicated that increases in egg weight caused by dietary fat are more pronounced during early egg production, i.e. <38 weeks old (Sell et al., 1987). The strain of the hens used in the experiment can also affect the response of egg weight to the fat tested, as demonstrated by Jackson et al. (1969) and Balnave (1982). In addition, the inclusion level of the dietary fat tested also affects the response (Menge et al., 1965a). All of these factors which are known to influence the effect of dietary fat upon egg weight differ between the studies cited in Table 1.2 and, hence, prevent precise comparisons.

General conclusions that might be drawn from the studies listed in Table 1.2 include:

Table 1.2: The range of reported egg weight responses to a variety of dietary fats

REFERENCE	STATUS OF LOW-FAT DIET: EFA-adequate	isocaloric	isonitrogenous	semi-purified	practical	Age (weeks)	EGG WEIGHT RESPONSE TO FAT SUPPLEMENT: Maize oil	Olive oil	Safflower oil	Soybean oil	Coconut oil	Sunflower oil	Arachis oil	Fish oil	Animal fat
Jensen et al. (1958)	Y	N	N	Y	N	38–43	+								+ + +
Shutze et al. (1958)	NA	NA	NA	NA	NA	NA	+		+	+					+ + +
Treat et al. (1960)	Y	Y	Y	Y	Y	32–64									+
Machlin and Dudley (1962)	NA	NA	NA	NA	NA	NA			+						
Jensen and Shutze (1963)	N	N	N	Y	N	25–65			+						
March and Biely (1963b)	Y	Y	N	N	Y	48–82									–
Marion and Edwards (1964)	N	Y	Y	Y	N	22–38	+				±			+ +	
Menge et al. (1965b)	N	N	N	Y	N	NA			+					+ +	
Balnave (1967)	Y	N	N	N	Y	52–60								–	
Balnave (1968a)	N	Y	Y	N	Y	52–57	+								
Balnave (1968b)	Y	Y	Y	N	Y	35–47	±								
Waring et al. (1968)	Y	N	N	N	Y	35–70							+		+
Cooper and Barnett (1968)	N	Y	Y	Y	Y	31–55	+								
Balnave (1970a)	N	Y	Y	N	Y	52–63	+							±	
Jackson et al. (1969)	Y	N	N	N	Y	26–62									+
Balnave (1972)	N	Y	Y	Y	N	30–62	+								
Whitehead (1981)	Y	Y	Y	N	Y	24–73	+	+							
Hoyle and Garlich (1987)	Y	N	N	N	Y	22–34				+					
Scragg et al. (1987)	Y	Y	Y	N	Y	22–69		+		+					
Balnave (1987)	Y	Y	Y	N	Y	38–50					–	+			

N: negative, Y: affirmative, NA: not available
+: egg weight increase, –: egg weight decrease, ±: no effect detected

i) unsaturated vegetable oils consistently increased egg weight

ii) saturated fats have been reported to increase or have no effect upon egg weight

iii) long chain polyunsaturated fatty acids have been reported to increase or decrease egg weight

iv) fat supplementation has increased egg weight and feed conversion efficiency without affecting feed intake or body weight.

It is now generally accepted that the addition to diets of some fats, especially vegetable oils, does increase egg weight, but the mechanism by which this phenomenon occurs is unknown. Fat has two important roles in nutrition: firstly, it is a highly concentrated form of metabolisable energy (ME); and, secondly, it is a source of essential fatty acids (EFAs). On this basis, past work has attempted to prove or disprove that dietary fat increases egg weight by providing additional ME or essential fatty acids.

a) Dietary fat as a source of energy

Egg weight has been reported to increase with high energy diets containing yellow grease (Hochreich et al., 1958), animal fat (Harms and Waldroup, 1961) and the methyl esters of cottonseed oil (Quisenberry and Bradley, 1962). However, no distinction could be made between the contribution of an energy source and the fat content per se from these studies. It is essential to separate the effect of increased energy concentration from a possible specific property of fat.

Unfortunately the early studies on the effect of dietary fat upon egg weight do not permit such a separation. Many workers (Treat et al., 1960; Shutze et al., 1962; Shutze and Jensen, 1963) replaced

the carbohydrate portion of the diet weight for weight with the fat in question. Fat contains over twice as much metabolisable energy (ME) as does the same weight of carbohydrate. Hence, these early studies compared diets differing in their ME content. Later studies used carefully formulated isoenergetic diets and in such cases dietary fat still increased egg weight (Marion and Edwards, 1964; Edwards and Morris, 1967; Cooper and Barnett, 1968). In an elegant experiment, Balnave (1971a) showed that egg size increased in fat-fed hens due to some component other than its ME content by manipulating just two components of the diet. To two identical diets he added equivalent levels of ME from maize starch or maize oil and reported that egg weight only increased in birds fed maize oil.

In the light of recent findings there must now be some doubt whether the above diets which were calculated to be isoenergetic were in fact equal in their ME content. Fat is the most difficult dietary component for which to determine an accurate ME value and Wiseman (1984) has recently reviewed the problem involved. There have been many reports of fats exhibiting ME contents far in excess of their calculated values. Jensen et al. (1970) reported yellow grease having approximately 32% "extra-calories" whilst Gomez and Polin (1974) reported the ME of fats actually exceeding their gross energy. The extra-caloric value of fats in poultry diets has recently been reviewed by Summers (1984). Dietary fats, particularly unsaturated fats, enhance the utilisation of other dietary components by increasing the absorption of saturated fatty acids (Young and Garrett, 1963) and reducing the rate of food passage (Mateos and Sell, 1981). Hence,

although workers such as Balnave (1971a) had formulated isoenergetic diets by calculating the sum of the individual dietary components' ME values, this will not have accounted for the associative effects of the fat with other dietary components.

Recently Whitehead (1981) has shown that a high-fat diet increases egg weight compared to a low-fat diet which was shown to be isoenergetic as determined by the true metabolisable energy (TME) assay developed by Sibbald (1976). This method of ME determination is the most accurate to date, and as the high-fat diet itself was assessed, any enhancement of the other dietary components by the fat would have been detected. Therefore, it can be concluded that the increase in egg weight produced by dietary fat appears to be an intrinsic property of the fat itself rather than a result of any increase in the energy concentration of the diet.

This conclusion is supported by consideration of the effect of ME upon egg weight. Investigators of the effect of dietary ME level on egg weight have generally formulated relatively high energy diets using dietary fat. Since it is supposed that dietary fats have an intrinsic property that is able to affect egg weight independently of their energy content (see above), it is not possible to separate the effects of dietary fat and ME level in many studies. The recommended ME content of practical layer diets is 11.1 MJ/kg (Bolton and Blair, 1977). Experimental designs that have varied dietary ME without changing fat levels have not shown that egg weight has been affected by energy concentrations slightly above or below the recommended amount. For example, Sadagopan et al. (1972) found no difference in egg weight or production using diets varying from 11.0-12.7 MJ/kg, nor did Saxena

et al. (1986) with values from 11.7-12.6 MJ/kg, nor did Sell et al. (1987) with ranges of 11.8-12.7 MJ/kg.

b) Dietary fat as a source of essential fatty acids

Another important function of dietary fat is as a source of essential fatty acids (EFAs). Animals can synthesise de novo fatty acids of the palmitoleate (n-7) and oleate (n-9) series, but the families based on linoleate (n-6) and linolenate (n-3) can be synthesised only if the basic fatty acids are provided in the diet. The subject of EFAs in poultry nutrition has been reviewed by Balnave (1970a) and more recently by Whitehead (1984) who stated that linoleic acid may be thought of as the quintessential fatty acid for poultry, since provision of it in adequate amounts eliminates all symptoms of EFA-deficiency. EFAs are vital for prostaglandin synthesis and are necessary for the formation of phospholipids.

Symptoms of EFA-deficiency in young chicks include retarded growth, increased water consumption, faulty feathering, and delayed development of secondary sex characteristics. Mature hens are difficult to deplete of EFAs because of the large reserves of linoleate present in adipose tissue. Therefore, unless animals are reared on diets low in linoleic acid, symptoms of EFA-deficiency may not be observed even after prolonged feeding of a linoleate-deficient diet. Menge et al. (1963) reported reduced egg production, egg size, fertility and almost total embryonic mortality from 45-week-old hens reared on an EFA-deficient diet since hatching compared to birds fed a practical diet. Thus, it was established that low egg weight was a symptom of EFA-deficiency in adult birds.

Initial studies on the relationship between dietary fat and egg size showed that maize oil and other vegetable oils high in linoleic acid content increased egg weight (Jensen et al., 1958; Shutze et al., 1959; Shutze et al., 1962; Machlin and Dudley, 1962). Shutze and Jensen (1963) reported that both methyl linoleate and a purified linoleic acid supplement added on an equivalent linoleic acid basis to 50 g maize oil/kg of feed resulted in similar egg weight improvements. From this study these authors concluded that linoleic acid was responsible for a major part of the egg weight effect obtained from feeding vegetable oils. This conclusion was supported by Marion and Edwards (1964) who investigated the response to a variety of fats and found that egg production, egg size and hatchability of eggs was positively correlated with the linoleic acid content of the diet. Hence, it was thought that the effect of dietary fat upon egg weight was due to the linoleic acid content. As a consequence, it was widely held that the laying hen had two requirements for linoleic acid - 9 g/kg of feed for physiological function and a higher requirement of 20-40 g/kg of feed for maximum egg size.

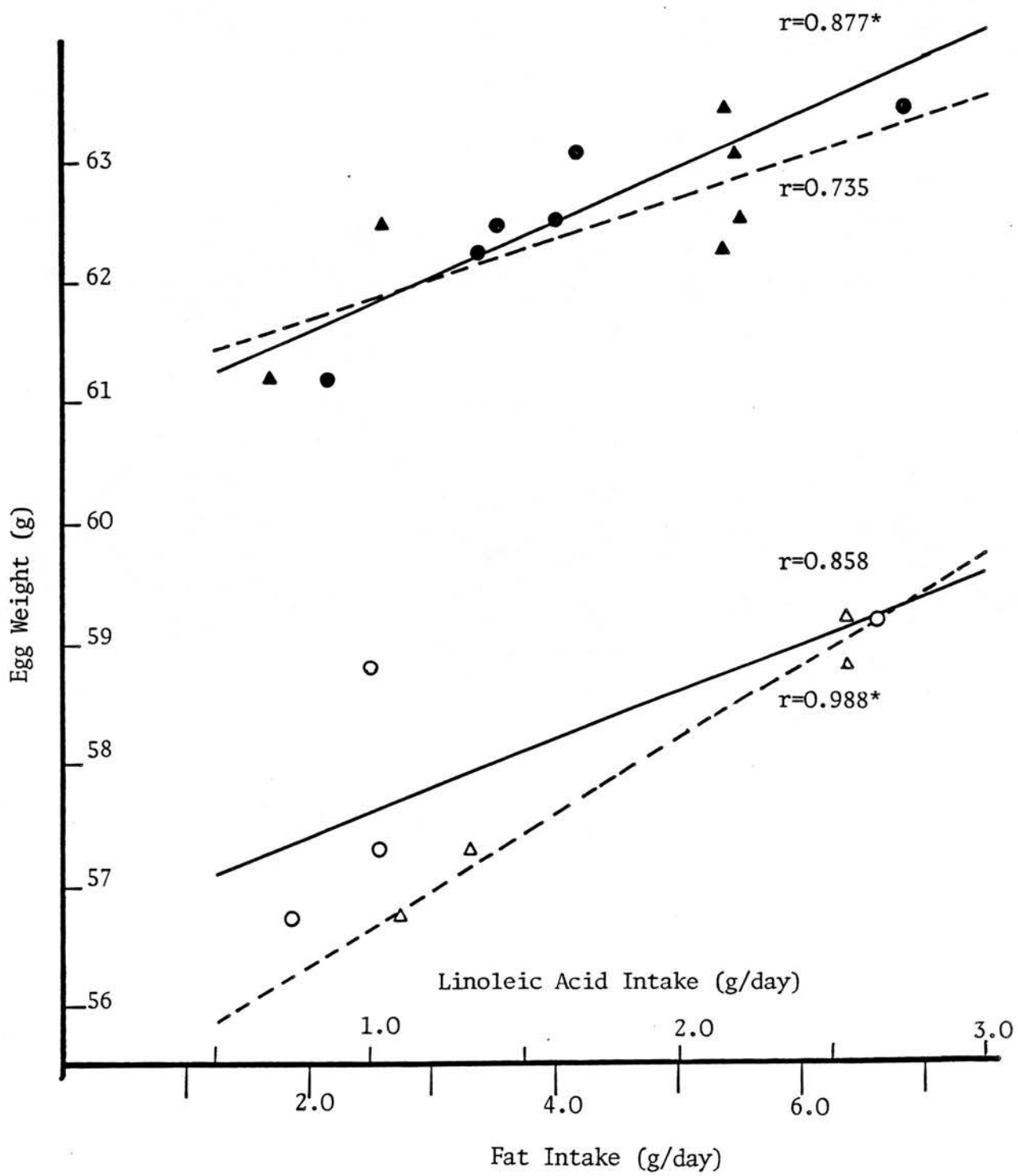
Although the above studies appeared to demonstrate that the response in egg weight to a fat supplement was due to its linoleic acid content, there are several reasons to query this assumption. The majority of the studies were conducted using EFA-deficient or severely EFA-depleted birds, and it is not surprising that egg performance was improved when fats high in linoleic acid were fed. In addition, Menge et al. (1965b) supplied equivalent amounts of linoleic acid in the form of safflower oil (77% linoleic acid) or menhaden oil (3% linoleic acid) and reported that the menhaden oil promoted an increase in egg weight

that could not be assigned to its linoleic acid content. Hence, it has not been conclusively demonstrated that the observed increase in egg size after fat supplementation was a unique response to linoleic acid rather than a response to fat in general or perhaps more specifically to unsaturated fat.

In an elegant experiment, Shannon and Whitehead (1974) attempted to resolve this problem. Hens were fed diets containing equal amounts of fat provided by varying proportions of safflower oil and olive oil (7% linoleic acid). All of the diets were isoenergetic, isonitrogenous and contained the same amount of total and unsaturated fat but differed in their linoleic acid content. No differences in egg numbers or egg weight were reported and the correlation between daily linoleic acid intake and egg weight was not significant (calculated from Shannon and Whitehead, 1974). Shannon and Whitehead (1974) concluded that linoleic acid in excess of the EFA requirement for normal functioning is not specifically required for maximum egg size. In a subsequent study, Whitehead (1981) showed that a diet containing 9 g linoleic acid/kg supplied by 30 g olive oil/kg caused a greater increase in egg weight than a diet containing the same amount of linoleic acid supplied by 4 g maize oil/kg, though egg weight was further improved by a diet containiing 23 g linoleic acid/kg supplied by 30 g maize oil/kg. There was a significant correlation between the daily intake of total fat and egg weight (Fig. 1.1, calculated from Whitehead, 1981), but no such correlation with the daily linoleic acid intake. Whitehead (1981) suggested that egg weight responds to higher dietary amounts of any readily absorbable fatty acids rather than to linoleic acid *per se*. Recently, Scragg *et al*. (1987) fed diets varying

Fig1.1:The relationship of mean egg weight with the daily intake of linoleic acid (circles joined by solid line) and with the daily intake of dietary fat (triangles joined by broken line). The data are calculated from Whitehead, 1981 (open symbols) and Scragg et al, 1987 (closed symbols).

(*, correlation coefficient significant at 5% level of probability)



in total fat and linoleic acid content using soybean oil, olive oil and two commercial fat blends. Egg weight was not correlated with daily fat intake (Fig. 1.1, calculated from Scragg et al., 1987), but there was a significant correlation with daily linoleic acid intake. Scragg et al. (1987) concluded that increasing the intake of readily absorbable fat without increasing linoleic acid intake does not increase egg weight. This is not in agreement with the findings of Whitehead (1981). However, careful examination of the data of Scragg et al. (1987) shows that some of the high fat diets increased egg weight without an increase in linoleic acid and that these responses just failed to attain statistical significance.

Although egg weight responds to dietary fat to the greatest extent in EFA-deficient hens and fats high in linoleic acid appear to be the most potent, even in EFA-adequate birds, there is sound evidence that non-EFAs can also exert an effect upon egg weight (Menge et al., 1965b; Shannon and Whitehead, 1974; Whitehead, 1981). Since non-EFAs cannot be converted to EFAs, it can be assumed that the increase in egg weight by dietary fat is not a result of increased EFA supply, but occurs by some other mechanism.

III. POSSIBLE MECHANISMS BY WHICH DIETARY FAT MIGHT AFFECT EGG SIZE

Though it is some 30 years since Jensen et al. (1958) reported that dietary fat is capable of increasing egg weight, the mechanism by which this phenomenon occurs has received scant attention.

There have been surprisingly few reliable reports on the relative changes in yolk, albumen and shell weight following fat supplementation. It would seem logical to expect that yolk would be

the most likely component to respond to dietary fat. Lipid makes up over a third of yolk on a wet weight basis and two-thirds on a dry matter basis, whilst neither albumen nor shell contain any lipid. The ratio of yolk weight to whole egg weight was found to be relatively constant in fat-fed birds (Jensen et al., 1958). Thus, they concluded that dietary fat increased egg weight via yolk rather than albumen or shell. Supportive evidence was provided by Jensen and Shutze (1963) and Balnave (1970b) who reported yolk weight increasing in EFA-deficient hens following fat supplementation. Sell et al. (1987) demonstrated a significant linear relationship between yolk weight and the fat level in the diet but no such relationship was detected for albumen weight.

EFAs are thought to be necessary in the transport of lipid (Whitehead, 1981). Jensen and Shutze (1963) speculated that the low yolk weight in EFA-deficient hens was due to a slower rate of lipid transport to the ovary and this might be reversed by dietary fat supplementation. This view is supported by the finding of Balnave (1970b) that the increase in yolk dry weight was due solely to an increase in the amount of lipid deposited in yolk. A variant of this mechanism was suggested for EFA-adequate hens. Bray (1967) attempted to restrict the ME intake of a maize oil-based diet so that body weight would be similar to that of pullets fed a basal diet. The maize oil-based diet was diluted with cellulose so that body weight was similar to that on the basal diet. Egg weight was still increased. However, if ME intake was restricted by allowing access to the maize oil-based diet for only 9 hours per day compared to 16 hours for pullets on the basal diet, egg weight was no longer increased. From

these observations, Bray (1967) suggested that the mechanism by which dietary fat increases egg weight might simply be one in which the rate of lipid deposition in the growing follicle is accelerated by the abundance of preformed fatty acids absorbed from the gut. Balnave (1971b) showed that there is a rapid deposition of dietary linoleate in the ovary and concluded that dietary lipid is deposited directly into the ovary. Indeed, the supposition that dietary fat improves egg weight via yolk weight by an increase in lipid deposition of dietary origin is so well accepted that it has been used to explain aspects of egg weight responses to dietary fat. Sell et al. (1987) suggested that the rate of synthesis of lipoproteins in young hens is insufficient to supply the amount of lipids needed for yolk deposition. In such cases, additional lipid in the form of dietary fat improves yolk weight. Older hens are proposed to have adequate rates of lipoprotein synthesis for optimum yolk development. Hence, this is the suggested reason that egg weight responds to dietary fat to a greater extent in younger birds (Sell et al., 1987).

Though this mechanism is capable of explaining several observations, it does not explain others and is open to several criticisms. For example, dietary fat has been noted to increase egg weight without affecting yolk size (Guenter et al., 1971; Herbert et al., 1987; Karunajeewa and Tham, 1987). Though the fatty acid composition of the yolk reflects that of the diet, many studies have shown that the total yolk lipid (g/g of yolk) appears virtually unaffected by the amount or type of fat in the diet (e.g. Combs and Helbacka, 1960; Balnave, 1967). Finally, there is strong evidence that dietary fat cannot be deposited directly into the yolk (Griffin and

Perry, 1985). Hence, it would appear that at present there is no satisfactory explanation for the effect of dietary fat upon egg weight.

IV. OBJECTIVES OF STUDY

The increases in egg weight due to dietary fat are commercially important, though they represent only 3-5% of the egg weight at best. Such small changes are difficult to study at the biochemical level and initially it is necessary to identify more specific aspects of the effect of dietary fat upon egg size. Determining which egg component responds to fat feeding would allow the targeting of biochemical investigations. The information gained during the investigation into the mechanism could then be used to formulate diets which would elicit responses in egg size that could be predicted, and accounted for, by the mechanisms proposed in this study.

To this end, the relative effect of a maize oil-supplemented diet upon yolk and albumen weight was investigated (Chapter 3). Information from this investigation was used to study the physiological basis of the mechanism of dietary fat upon egg weight (Chapter 4). Finally, egg weight responses to a variety of commonly used fat types fed at several levels of inclusion were investigated (Chapter 5).

V. EGG PRODUCTION: BACKGROUND INFORMATION

To aid the reader not acquainted with the poultry industry, a brief account of the life history of the battery hen and of the biology and physiology of egg formation is provided in the following sections.

A. The Life history of the Battery Hen

It is generally assumed that the domestic chicken originated from one or all four species of Jungle Fowl. However, whereas in the past, dual purpose-type fowl lines were common in which females were retained for laying and the males fattened for table, there is now a clear distinction between birds kept for egg production (layers) and those kept for meat production (broilers).

The laying hen egg is incubated for 21 days under controlled temperatures and humidities. Chicks for the first 3 weeks of their lives are provided with artificial heat, until the birds have developed some feathers. At about 6 weeks old the chicks are placed in tiered wire cages and reared there until just before they come into lay when they are placed in laying battery cages, the most common method of housing laying hens. In these batteries, the birds are kept, usually in ones, twos or threes, in metal wire cages erected in tiers in well-ventilated buildings. The floors on which the birds stand are of wire mesh so that their excreta fall through on to a tray below from which they are removed by some type of mechanical cleaning device. The mesh floors slope so that the eggs run down into a collecting trough in front. The birds are placed in the cages just before they come into lay at about 18 weeks of age, and remain in the cages for about 12 months, when they are sold as boiling fowls for table purposes.

The age at which fowls come into lay varies according to the strain, the level of nutrition during rearing and the lighting pattern. Typically, the hours of light are 10 hours per day at 16 weeks old, then this increases by 30 minutes per week until 17 hours of light are provided each day. This increase in daylength affects the neuro-

endocrine control of sexual maturity and ovulation resulting in the majority of hens coming into lay at about 22 weeks of age.

When production commences, hens lay relatively small eggs, the eggs increasing in weight during the laying season (typically 50 weeks). However, as the season progresses, the hen lays fewer eggs until production ceases completely as the bird begins to moult. Hens that are retained for the second season produce approximately 15% fewer eggs than in their first season. Consequently, most fowl kept for egg production are slaughtered when they are about 18 months old and replaced with younger hens.

B. Egg Formation

The following description of the physiological processes involved in egg formation is only a brief review and the reader is directed to the excellent reviews of Griffin et al. (1984) and Tullett (1985) for further reading on yolk and shell formation, respectively. Albumen formation is a less well understood process (see Gilbert, 1976).

The organs directly engaged in the production of eggs are the ovary (responsible for yolk formation) and the oviduct (responsible for albumen and shell formation). Only the left ovary and oviduct develop in the hen, the right ovary and oviduct being either absent or rudimentary. As the ovary develops and secretes oestrogen and progesterone (see Wells and Gilbert, 1984) profound changes occur in the blood chemistry. Many of the yolk precursors including vitellogenin and very low density lipoprotein (VLDL) are synthesised by the liver as the plasma oestrogen concentration increases. The ovary

possesses a series of yolk-filled oocytes in different stages of development. Each develops from a single cell, the ovum, and at first (up to 5 mm diameter) growth is slow. About 8 to 10 days before the first egg is laid the yolks begin to increase in size rapidly as yolk precursors are taken up by receptor-mediated endocytosis, but successively, and the final size of about 32 mm diameter is reached in about 7 days. At this stage the yolk follicle ruptures and the yolk is released into the body cavity. About 6-7 follicles are in the final stage at rapid growth at any one time.

The ovulated yolk is engulfed by the proximal end of the oviduct, the infundibulum, and spends about 0.5 hours travelling through this region during which time a thick layer of albumen, rich in the protein ovomucin, is added around the yolk. As the egg travels down the oviduct it rotates and spins the fibres of ovomucin into two ropes, the chalazae, which support the yolk to both poles of the egg. After leaving the infundibulum, the yolk passes into the magnum which represents nearly half the total length of the oviduct. The magnum is responsible for the synthesis, storage and secretion of all 40 or so individual proteins comprising the albumen (O'Malley et al., 1969, and Gilbert, 1976). After oestrogen stimulation, the mucosa of the magnum differentiates into three distinct cell types: a ciliated cell-type concerned with motility; and two albumen protein synthesising cell-types, the tubular gland cell and the goblet cell (Schimke et al., 1973). Under the influence of oestrogen, the tubular gland cell produces most of the albumen proteins including the cell-specific proteins ovalbumin and lysozyme, whilst the goblet cell is stimulated by progesterone to produce just one protein, avidin. By the time the

yolk leaves the magnum (about 3 hours), its complement of albumen proteins has been laid around it, but at this stage the albumen is in the form of a concentrated protein and represents only half the volume of the albumen in the freshly laid egg.

The developing egg passes from the magnum and into the isthmus where there is a rapid development of two shell membranes around the albumen. During the passage through the isthmus (about 1.25 hours), water containing various salts passes through the membrane "plumping" the egg albumen. This "plumping" process continues in the shell gland until the albumen contains its full complement of water, about 87% (w/w).

Shell formation, which occurs in the shell gland, is a slow process and takes nearly 21 hours. Mamillary cores are deposited on the outer shell membrane and act as sites for subsequent calcium carbonate crystallisation which forms the true shell. Pigment, mainly protoporphyrin, is deposited on to the crystal surface and finally the whole egg is enveloped in a thin cuticle of protein before being laid (oviposition).

After oviposition, the next yolk is ovulated approximately 30 minutes later. Hens lay a number of eggs on consecutive days before a rest day, these eggs constitute a sequence or a clutch. The number of eggs in a sequence varies enormously from 1 to 300, but is typically 7.

CHAPTER 2

MATERIALS AND METHODS

I. ANIMAL HUSBANDRY

All of the birds used in the study were a commercial layer strain, Isabrown.

A. Rearing

Husbandry

The birds were obtained as day old chicks and reared according to standard pullet replacement procedures up to 17 weeks old. The day old chicks were placed in heated brooder cages until 4 weeks of age then transferred to cage pens in housing kept at a temperature of 22-23°C. At 14 weeks of age the hens were placed in individual cages (46 cm x 30 cm x 40 cm) in three-tier Thornber battery units.

Throughout the rearing stages, the photoperiod was 8L:16D. This was increased at 14 weeks of age to 14L:10D in all experiments, unless otherwise stated.

Diets

The birds were fed a standard chick grower diet (approximately 200 g/kg crude protein and 25 g/kg fat) until 6 weeks old and then a standard rearer diet (approximately 150 g/kg crude protein and 25 g/kg fat) up to 16 weeks of age. At 17 weeks the birds were transferred either to a standard layer diet (approximately 150 g/kg crude protein and 25 g/kg fat) or to one of several experimental diets.

All diets were in the form of mash and formulated to supply all the essential nutrients. Food and water were always available ad libitum.

B. Diet formulation

The experimental layer diets were formulated with the aid of the computer programme FORMULATE which was supported on a Prime 550 mainframe computer. The programme contained the chemical analyses of all the dietary components used and compared the individual nutrients within a formulated diet with the recommended level of nutrients for a layer diet.

The diets were mixed in a commercial-type mill and samples were taken for the analysis of the basic components by the methods described in the Fertilizers and Feeding Stuffs Act (1982).

II. MEASUREMENTS OF EGG CHARACTERISTICS

A. Egg weight

Egg weights were recorded by means of an electronic balance (Sartorius MP) connected to a microcomputer (Epsom HX-20). A computer programme, EGGWT, written by Mr J. Wright, enabled the recording of the bird identification number, number of whole eggs, number of other eggs, and the egg weight (to 1 decimal place). Data recorded on the microcomputer was then transferred to a Prime 550 mainframe computer for analysis.

B. Internal components

Yolk and albumen wet weights

The yolk was separated from the albumen with a domestic egg separator. Any adhering albumen was removed from the yolk by rolling on adsorbant paper. Yolk and albumen weight were recorded (to 1 decimal

place) using an electronic balance. The shell weight was calculated by subtraction.

Yolk and albumen dry weight

For each bird, the yolk and albumen separated from 3 eggs were pooled and mixed. Approximately 20.0 g (weighed to 3 decimal places) of yolk and albumen were placed in pre-weighed aluminium dishes and frozen. The yolk and albumen samples were then freeze-dried for 7 days and re-weighed.

III. BLOOD COLLECTION

Immature broiler serum

Birds aged less than 10 weeks old were used to obtain large quantities of serum which was employed in the activation of substrate in the lipoprotein lipase (Lpl) assay (see below).

A lethal dose of sodium pentobarbitone (Expiral; Abbott Laboratories, Kent) was administered to the bird via the brachial vein. Feathers and skin were removed to expose the jugular vein and, with the bird held above a beaker standing in ice, the vein was cut. Several birds were exsanguinated in this manner and the blood pooled.

The blood was allowed to clot for several hours at room temperature and stored overnight at 4°C. Serum was recovered from the clot by centrifuging at 2,500 g for 10 minutes and stored at -20°C in several aliquots.

Hen plasma samples

Normal plasma samples

Laying hen blood samples, for various studies, were taken by venepuncture of the brachial vein. With both feet held together, the bird was laid on its side and its wing outstretched to expose the undersurface. The skin above the brachial vein was plucked free of feathers and a blood sample, up to 7 ml, was taken using a disposable needle and syringe. The blood sample was then placed in a tube containing enough 0.2 M EDTA, pH 7.4 to give a final concentration of approximately 7.5 mM EDTA.

Plasma was recovered by centrifugation at 1,000 g for 10 minutes at 4°C, and stored at -20°C until required.

Post-heparin plasma samples

Blood samples taken after an intravenous injection of heparin solution were used to measure the functional lipase activity in birds (see Cryer, 1981 and Chapter 4).

A solution of the sodium salt of porcine heparin (Sigma) was made up in 0.9% saline at a concentration of 1,000 IU/ml. 500 IU/kg body weight was administered via the brachial vein. Exactly 2 minutes later a blood sample was taken from the opposite wing and plasma was collected as before. The plasma was kept ice-cold until assayed on the same day for total lipase and hepatic lipase activity.

IV. LIPOPROTEIN SEPARATIONS

A. Isolation of lipoproteins from plasma

Portomicrons

Portomicrons, lipoproteins of intestinal origin, were isolated from plasma by ultracentrifugation. Plasma in 16.5 ml centrifuge tubes was overlaid with at least 5 ml 0.9% saline and then centrifuged at 110,000 g at 10°C for 1 hour in a swing-out bucket rotor. The solid white plug at the surface and the creamy liquid a few millimetres below were removed and resuspended in 0.9% saline with a glass/Teflon homogeniser.

Very low density lipoproteins

Very low density lipoproteins (VLDL) were isolated from plasma by differential ultracentrifugation. The plasma was treated as above, portomicrons removed and the overlay readjusted with more 0.9% saline. The samples were recentrifuged at 110,000 g at 10°C for a further 20 hours and the solid yellow plug at the surface was removed and resuspended in 0.9% saline with a glass/Teflon homogeniser.

Portomicrons and very low density lipoproteins

Lipoproteins of relative density <1.006, containing both portomicrons and VLDL, were isolated from plasma by ultracentrifugation. The plasma was overlaid as above and centrifuged at 110,000 g at 10°C for 20 hours. The portomicron/VLDL surface plug was removed and resuspended in 0.9% saline with a glass/Teflon homogeniser.

B. Gel filtration of lipoproteins

Lipoproteins isolated by centrifugation were made up to 2 ml with eluting buffer (0.154 M NaCl, 20 mM Tris/HCl, 1 mM EDTA; pH 7.6 containing 0.01% sodium azide) and loaded onto a Bio-Gel A-150m (Bio-Rad Laboratories, Watford, UK) column (43 x 2.6 cm) which had been equilibrated with the same buffer. The column was eluted with the same buffer at a flow rate of 10 ml/hour. Fractions of (2-3 ml) were collected and assayed for the presence of lipoproteins by measuring the absorbance at 280 nm, or by liquid scintillation counting if appropriate.

V. LIPID EXTRACTION

A. Yolk samples

Total yolk lipid was determined gravimetrically by a two-step Folch extraction as modified by Smith et al. (1964) and applied to yolk lipid (Fletcher et al., 1984). In this latter study, Fletcher et al., assessed the precision of several methods of yolk lipid determination and reported the two-step Folch extraction method to have the lowest coefficient of variation (2.1%).

Approximately 0.5 g yolk samples were weighed (to 3 decimal places) in glass-stoppered test tubes, mixed with 0.5 ml 8.8 g KCl/l. and stored at -20°C until required.

The yolk samples were thoroughly mixed with 10 ml chloroform: methanol (2:1 v/v) and allowed to stand for 30 minutes. After centrifuging at 500 g for 10 minutes, the supernatant was filtered through scintered glass filter funnels. The centrifuged residue was

re-extracted with a further 10 ml chloroform:methanol (2:1 v/v) and filtered. The combined filtrates were thoroughly mixed with 4.5 ml 8.8 g/l KCl. The two phases were separated by centrifugation at 500 g for 10 minutes and the upper aqueous phase was aspirated. Enough methanol was added to the lower phase to produce a single phase and the lipid was evaporated to dryness at 40°C under a gentle stream of nitrogen. The dried lipid extract was transferred with 10 ml chloroform:methanol (19:1 v/v) into glass vials, pre-weighed to 3 decimal places, and dried to a constant weight under a gentle stream of nitrogen.

B. Tissue samples

Lipids were extracted from tissues using a modification of the method described by Folch et al. (1957).

The frozen tissues were placed in liquid nitrogen and then crushed immediately. Approximately 1.0 g of tissue (0.5 g for liver, adipose and follicles) was weighed, to 3 decimal places, in stoppered boiling tubes. 15 ml chloroform:methanol (2:1 v/v) was added and homogenised with a Polytron tissue disintegrator (Kinematica, Lucerne, Switzerland). The mixture was allowed to stand for 30 minutes with occasional mixing and then filtered through scintered glass filter funnels. A further 5 ml chloroform:methanol (2:1 v/v) was used to rinse the original test tube and filter funnel. 3.75 ml 0.9% saline was added and the tube was thoroughly shaken. The upper aqueous phase was aspirated after centrifugation at 500 g for 10 minutes. Enough methanol was added to the lower phase to produce a single phase and solvent was removed in a vacuum oven at 45°C.

The extracted tissue lipids were stored at -20°C until required.

C. Aqueous samples

Lipid was extracted from aqueous samples by the method of Bligh and Dyer (1959).

For aqueous samples of volumes up to 1 ml, 3 ml chloroform: methanol (1:2 v/v) was mixed with the sample followed by the addition and mixing of 1 ml 0.9% saline. A further 2 ml chloroform was added and the two phases were separated by centrifugation at 1,000 g for 10 minutes. The upper phase was aspirated and the lower phase was washed with 2 ml synthetic upper phase and separated, as above. Enough methanol was added to the lower phase to produce a homogenous phase and solvent was evaporated at 40°C under a gentle stream of nitrogen.

VI. TRIGLYCERIDE DETERMINATION

Triglyceride concentrations were determined according to a modified method of Kessler and Lederer (1965) following lipid extraction by the method of Bligh and Dyer (1959).

The extracted lipid was resuspended in a suitable volume of isopropanol. Duplicate aliquots, estimated to contain 0-20 μmol triglyceride, were dispensed into test tubes and the volumes were made up to 6.0 ml with isopropanol. A range of standards, 0-2.0 μmol, were similarly prepared from a stock solution of 1 μmol/ml triolein in isopropanol and treated in an identical manner as the unknowns.

Approximately 2 g activated silicic acid (Sil-A-200, Sigma) was mixed with each sample and allowed to stand at room temperature for 5 minutes. The tubes were shaken and centrifuged at 2,000 g for 10 minutes. 3 ml supernatant aliquots were transferred to glass stoppered test tubes and 1.0 ml KOH (20 g/l) was added with immediate mixing.

The samples were heated at 50°C in a water-bath for 10 minutes. After cooling, 0.6 ml periodate reagent (5.4 g sodium periodate and 115 ml glacial acetic acid made up to 1 litre with distilled water) was added and mixed immediately. Then, 1 ml freshly prepared acetylacetone reagent (75 μl acetylacetone and 250 μl isopropanol made up to 100 ml with 2 M ammonium acetate, pH 6.0) was added with immediate mixing and the solution was heated, as before.

The samples were kept in the dark until cool and shaken before the absorbance was determined at 440 nm.

VII. PROTEIN DETERMINATION

The protein concentrations were determined by several methods depending upon the protein concentration of the sample and degree of accuracy required.

A. Absorbance at 280 nm

The absorbance of a protein solution at 280 nm was the simplest and most rapid method for protein estimation. The spectrophotometer was zeroed against the buffer in which the samples were dissolved and the samples were further diluted in this buffer, if required.

B. Colorimetric Methods

Lowry Protein Determination

The method of protein determination of Lowry et al. (1951) was employed for samples containing protein in the range 0-1 mg/l.

The reagent solution was prepared daily from stock solutions by the addition of 1 ml 1% (w/v) $CuSO_4.5H_2O$ and 1 ml 2% (w/v) sodium potassium tartrate to 100 ml 2% (w/v) Na_2CO_3 in 0.4% (w/v) NaOH.

The protein sample (200 μl) was pipetted into the test tube and the volume was made up to 200 μl with 0.9% saline. A range of protein standards, 0-200 μg, were similarly prepared from a stock solution of 1 mg/ml bovine serum albumin (Cohn Fraction V, Sigma) dissolved in 0.9% saline and treated in an identical manner as the unknowns. To each sample, 2.0 ml reagent solution was added. After 10 minutes, 200 μl 50% (v/v) Folin-Ciocalteu reagent (Sigma) was added and shaken immediately. The optical density at 720 nm was measured after 20 minutes at room temperature.

Bradford Protein Determination

The protein assay method of Bradford (1976) was employed for samples with protein concentrations in the range 0-0.1 mg/ml.

The protein reagent was prepared by disolving 100 mg Page Blue G-90 (Sigma) in 100 ml 85% (w/v) H_3PO_4. 50 ml 95% (v/v) ethanol was added and the solution was made up to 1 litre with constant stirring. The solution was filtered through Whatman No.1 filter paper and stored in the dark.

Protein samples (0-10 μg, were similarly prepared from a stock solution of 100 μg/ml bovine serum albumin (Cohn Fraction V, Sigma) dissolved in 0.9% saline and treated in an identical manner as the samples. To each sample was added 1 ml protein reagent. The optical density was measured at 595 nm after 10 minutes.

C. Nitrogen Determination - Kjeldahl

The protein content of egg albumen was determined by the standard Kjeldahl method.

The freeze-dried albumen sample (see above) was thoroughly mixed and approximately 0.3 g (weighed to 4 decimal places) was placed

at the bottom of a 100 ml digestion tube. One copper and one selenium Kjeldahl catalyst tablet was added together with 10 ml concentrated H_2SO_4. The digestion tubes were fitted to a condenser manifold and heated to 300°C on a dry block for 30 minutes.

After the tube had cooled, it was connected to a distillation system (Buchi, Model 321) and approximately 80 ml distilled water was added. Enough 10 M NaOH was added to turn the sample solution brown. Steam from the distillation system was passed through the sample solution and approximately 150 ml distillate was collected in a 250 ml conical flask containing 20 ml 4% (w/v) boric acid and 4 drops of Tashiro's Indicator.

The ammonia carried over by the distillate was titrated with 0.1 M HCl and the nitrogen content was calculated. A factor of 6.25 was used in converting the nitrogen content to the crude protein contained in the dried albumen sample.

VIII. THE DETERMINATION OF LIPOPROTEIN LIPASE ACTIVITY

Lipoprotein lipase (clearing factor, EC3.1.1.34) activity was determined by the hydrolysis of triglyceride and the subsequent measurement of the fatty acids released.

A. Substrate

The substrate used in the assay of lipoprotein lipase (Lpl) was Intralipid (20% Kabi Vitrum, Stockholm, Sweden), a triacylglycerol emulsion. The free fatty acid and phospholipid content of the Intralipid was reduced by treatment with defatted bovine serum albumin. Purified Intralipid was activated by pre-incubation with immature broiler serum that acted as a source of Lpl-activator apoprotein.

Preparation of de-fatted Bovine Serum Albumin.

Fatty acids were removed from bovine serum albumin (BSA) by treatment with charcoal according to the method of Chen (1967).

Fatty acid poor BSA (Cohn Fraction V, Sigma) was dissolved in distilled water at 23°C to make a 10% (w/v) solution. Activated charcoal powder(0.5 g/g BSA) was added and the pH was lowered to 3.0 by the addition of 0.2 M HCl. After stirring for 1 hour at 0°C, the charcoal was removed by centrifugation in a swing-out bucket rotor at 20,000 g and 2°C for 20 minutes. Fine particles of charcoal were removed by filtration through a glass fibre disc (Whatman GF/D) under vacuum. The solution was brought to pH 7.0 by the addition of 0.2 M NaOH and then dialysed exhaustively against distilled water at 4°C.

After lyophilisation, the defatted BSA was used in the purification of the Intralipid substrate and in the Lpl assay buffer (see below).

Purification of Intralipid

The Lpl substrate, Intralipid, was incubated with defatted BSA to reduce the amount of endogenous free fatty acids and phospholipids present.

One volume of Intralipid was incubated with 4 volumes of 250 mM Tris/HCl, pH 8.0 containing 10 mM $CaCl_2$ and 2.5% (w/v) defatted BSA at 37°C for 30 minutes. The Intralipid was recovered by centrifuging at 50,000 g in a swing-out bucket rotor for 20 minutes at 20°C, and resuspended in 0.9% saline to the original Intralipid volume. The free fatty acids and phospholipids remained in the infranatant.

Purified Intralipid was prepared weekly and kept at 0°C.

Intralipid Activation

Immediately before the Lpl assay, the purified Intralipid was activated by the formation of triacylglycerol-rich lipoproteins containing the Lpl-activator apoprotein.

Three volumes of immature hen serum were incubated with 1 volume of purified Intralipid in a shaking water-bath at 37°C for 1 hour. The activated substrate was kept at 0°C before use on the same day.

B. Lipoprotein Lipase Activity Assay

Determination of the Lpl activity for a given enzyme source was performed at four concentrations of the enzyme source, each in duplicate. The linearity (amount of enzyme source α enzyme activity) was investigated to establish that the assay was valid.

An assay volume of 250 μl was usually employed including 50 μl activated substrate (approximately 12 μmol triacylglycerol) and 50 μl assay buffer 250 mM Tris/HCl, 10 mM $CaCl_2$, pH 8.2 containing 10% BSA. For each enzyme source various volumes (1-150 μl), usually in duplicate, were added and the volume made up with the appropriate enzyme diluent. The final assay volume was 250 μl with a final concentration of 50 mM Tris/HCl, 2 mM $CaCl_2$, and 2% BSA.

The assay mixture was incubated in a shaking water-bath at 37°C for 1 hour. The reaction was stopped by the addition of 2.4 ml ice-cold 0.1 M glycine pH 2.7, and the assay tubes stored at -20°C.

C. Semi-Automated Method for Free Fatty Acid Determination

The free fatty acids released by Lpl were extracted from the incubation mixture and their concentration was determined by a copper soap method based on that of Duncombe (1962) and adapted for automated analysis by Bowyer et al. (1978).

Free fatty acids were extracted from the aqueous phase by the addition of 3.2 ml di-n-butyl ether:ethanol:heptane (250:150:75 v/v/v) to the assay mixture and vortexing thoroughly for 3 x 1 minute, with 5 minutes between each vortexing. The aqueous and organic phases were separated by centrifuging at 1,000 g for 10 minutes. The upper di-n-butyl ether phase containing the free fatty acids was loaded into glass sample cups on a Skalaar Autoanalyser.

The free fatty acids were determined by a continuous flow method according to a modified method of Bowyer et al. (1978). The copper reagent was made daily by mixing equal volumes of 0.9% (w/v) $CuSO_4.5H_2O$ solution and a solution containing 100 g NaCl, 40 ml glycerol, 40 ml piperidine, 5 ml glacial acetic acid and 600 ml distilled water. As the extracted free fatty acids passed through the Autoanalyser, copper soaps were formed with the copper reagent. The amount of copper, in the form of soaps entering the organic phase, was determined by its reaction with 0.3% (w/v) 1,5-diphenylcarbazide, 0.5% (v/v) glacial acetic acid in isopropanol. The absorption at 550 nm was recorded on a chart recorder. The heights of the peaks were proportional to the fatty acid concentration and were compared to palmitic acid standards (0-0.5 μmol/tube) which were prepared in a similar manner to the unknowns.

IX. TISSUE LIPOPROTEIN LIPASE ACTIVITY

The Lpl activity of various tissues was determined following the preparation of acetone-ether powders. Acetone-ether powders concentrate and delipidate the enzyme whilst inactivating any hormone sensitive lipase activity.

Birds were intravenously administered a lethal dose of sodium pentobarbitone and the tissues to be studied were rapidly removed and rinsed in ice-cold 0.9% saline. After blotting dry and weighing, the tissues were frozen in liquid nitrogen and stored at -80°C.

The tissues were homogenised with a Polytron tissue disintegrator (Kinematica, Lucerne, Switzerland) in ice-cold water containing 20 μg heparin/ml (3 Units/ml) at 0°C (10 ml/g of tissue). The homogenate was allowed to extract for 30 minutes and then rehomogenised. Duplicate 2.0 ml aliquots of the homogenate were dispensed and 100 μl 10% (w/v) bovine serum albumin was added as protein carrier. 10 ml ice-cold acetone was added, vortexed and allowed to stand at 0°C for a further 30 minutes. The precipitated protein was pelleted by centrifugation at 1,000 g at 0°C for 10 minutes. The precipitate was washed with a further 10 ml ice-cold acetone and then with 10 ml ice-cold diethyl ether. Residual ether was removed under a gentle stream of nitrogen at room temperature and the acetone-ether powder stored at -70°C.

Extraction of the enzyme from the acetone-ether powder was achieved by homogenisation in 2 ml 10 mM Tris/HCl, pH 8.0 containing 20 μg heparin/ml using a Polytron homogeniser. After standing at 0°C for 1 hour, the extracted solution was used as an enzyme source in the Lpl assay.

X. POST-HEPARIN PLASMA LIPASE ACTIVITY

Lipoprotein lipase is thought to be synthesised in a variety of tissues and then secreted and transported to its functional location, the luminal surface of the capillary endothelial cells in those tissues

(see Cryer, 1981). The enzyme is anchored to the luminal surface of the capillary by glycosaminoglycan chains and in this position it can interact with triglyceride-rich lipoproteins present in the plasma. Displacement of the glycosaminoglycan chains by intravenous injection of heparin releases Lpl and hepatic lipase into the general circulation (see Cryer, 1981). In this manner, post-heparin plasma provides a measurement of total functional lipase activity.

Immunoprecipitation of Lpl with a specific antibody allowed the contribution of Lpl to the total lipase activity to be distinguished from hepatic lipase activity.

Total Lipase Activity

Post-heparin plasma samples were obtained as described above. The plasma was diluted 1:4 with 0.9% NaCl and assayed for lipase activity as described for Lpl activity.

Hepatic Lipase Activity

The sheep anti-hen lipoprotein lipase serum (see below) was diluted 1:20 with 0.9% NaCl. 300 μl diluted post-heparin plasma was incubated with an equal volume of diluted antiserum at 4°C for 30 minutes. Precipitation of the enzyme-antibody complex was aided by the addition of 30 μl donkey anti-sheep IgG antiserum (Scottish Antibody Production Unit, Carluke, Scotland) and incubated at 4°C for a further 30 minutes. After centrifuging at 1,500 g at 4°C for 30 minutes, the supernatant was assayed for residual lipase activity.

Lpl activity was calculated to be the difference between the total lipase and the hepatic lipase.

XI. POLYCLONAL SHEEP ANTI-HEN LIPOPROTEIN LIPASE ANTIBODY PRODUCTION

A polyclonal antibody to Lpl was raised in a sheep by immunisation with hen adipose Lpl purified from acetone-ether powders by two-step affinity chromatography, essentially as described by Bensadoun and Kompiang (1979). This antiserum was used to prepare labelled lipoproteins and to distinguish Lpl from other lipase activity.

A. Purification of Lipoprotein Lipase

(i) Preparation of Acetone-Ether Powders

Abdominal fat pads were removed from birds aged 3-7 weeks old, rinsed in ice-cold saline, blotted dry and then plunged in liquid nitrogen. The adipose tissue was stored at -80°C until enough material was obtained, typically 500 grams.

Approximately 200 g aliquots of adipose tissue were placed in liquid nitrogen and crushed whilst brittle. The adipose tissue powder was homogenised with a Polytron tissue disintegrator (Kinematica, Lucerne, Switzerland) in 5 volumes of ice-cold water and left at 0°C for 30 minutes. Following a further rehomogenisation, the floating fat was removed from the liquor by filtration through loosely-packed glass wool. Bovine serum albumin (5 mg/ml filtrate) was added as carrier and then 10 ml ice-cold acetone per ml filtrate. The mixture was left to stand for 30 minutes at 0°C with occasional stirring and then filtered under vacuum through Whatman No. 1 filter paper. The trapped residue was washed twice with 200 ml ice-cold acetone and twice with 200 ml diethyl ether. The acetone-ether powder was thoroughly dried in air and the powder removed and stored at -80°C.

(ii) Two-Step Affinity Chromatography of Lipoprotein Lipase

All operations during the purification of Lpl were carried out at 0-4°C. The buffer used contained 30% (w/v) glycerol and 10 mM sodium phosphate, pH 6.5, though the NaCl concentration was varied (see below).

Heparin-Sepharose CL-6B Affinity Chromatography

The acetone-ether powder was extracted in ice-cold buffer, containing 1.2 M NaCl, at a concentration of 75 mg/ml using a Polytron homogeniser. After leaving for 1 hour at 0°C, the mixture was centrifuged at 25,000 g at 0°C for 20 minutes. The supernatant was dialysed at 4°C overnight against 2 volumes of buffer to bring the concentration to 0.4 M with respect to NaCl.

The dialysed extract was filtered through a Whatman glass fibre filter (GFA) and then loaded onto a Heparin-Sepharose Cl-6B (Pharmacia, Uppsala, Sweden) column (11 x 3 cm) equilibrated with buffer, containing 0.4 M NaCl, at a rate of about 40 ml/hour. The column was then washed with buffer, containing 0.5 M NaCl, until no more protein was eluted, as determined by the Bradford protein assay. The enzyme was eluted with a linear gradient of NaCl concentration from 0.5 M NaCl to 1.5 NaCl formed by mixing 25 ml of both buffers. 2 ml fractions were collected and assayed for Lpl activity.

Fractions containing the major peak of enzyme activity were pooled and dialysed against the buffer to reduce NaCl concentration.

Concanavalin-A-Agarose Affinity Chromatography

The enzyme preparation was adjusted to 1.0 M NaCl by the addition of buffer containinig 3.0 M NaCl and loaded at a rate of 10 ml/hour onto a Concanavalin-A-Agarose (Sigma) column (5 x 1.5 cm)

which had previously been equilibrated with buffer containing 1.0 M NaCl. The column was then washed with the same buffer until no more protein is eluted, as determined by the Bradford protein assay. The enzyme was eluted using buffer containing 1.0 M NaCl and 0.2 M α-methyl-D-mannoside (Sigma). 1 ml fractions were collected and assayed for Lpl activity.

Fractions containing the major peak of enzyme activity were pooled and dialysed exhaustively against several changes of distilled water at 4°C and then lyophilised.

B. Raising Anti-Lipoprotein Lipase Antibody

Immunisation

The lyophilised Lpl was resuspended in 4 ml water and emulsified with an equal volume of Freund's complete adjuvant (Sigma). One Merino sheep was injected intramuscularly at several sites in the adductor muscles with the enzyme/adjuvant preparation. Booster immunisations using Freund's incomplete adjuvant were performed 21 days after the primary immunisation and fortnightly thereafter until the desired antibody titre was achieved.

Preparation of Antiserum

Small volumes of blood needed to monitor the antibody titre were taken from the ear vein of the sheep. Large volumes (up to 400 ml) of blood were taken from the jugular vein when a high antibody titre had been established. The blood was allowed to clot at room temperature for about 1 hour and then left at 4°C overnight for the clot to retract. Serum was recovered from the clot by centrifugation at 2,500 g at 4°C for 10 minutes and stored at -20°C.

C. Purification of Anti-Lipoprotein Lipase Immunoglobulin IgG

It was necessary to isolate the immunoglobulin IgG from the antisera for in vivo studies. IgG was isolated from large volumes of sheep serum by ammonium sulphate precipitation followed by negative adsorption on diethylaminoethyl cellulose 52 (DEAE-52) as described by Butterwith (1984).

Ammonium sulphate was slowly added to the sheep serum at 4°C up to a concentration of 291 g/litre. After centrifugation at 10,000 g at 4°C for 10 minutes, the precipitate was washed 5 times with 1.75 M ammonium sulphate at 4°C followed by centrifugation at 12,000 g at 4°C for 10 minutes each time. The washed precipitate was dissolved in 10 mM sodium phosphate, pH 7.0 and dialysed against distilled water at 4°C overnight. After centrifugation at 10,000 g at 4°C for 10 minutes the supernatant containing immunoglobulin was decanted from the lipoprotein precipitate and dialysed against 10 mM sodium phosphate, pH 8.0 at 4°C overnight.

DEAE-52 (Whatman) was stirred with 5 volumes of 0.5 M HCl for 30 minutes. It was then filtered and washed with distilled water until the effluent had a pH 4.0. The gel was stirred with 5 volumes 0.5 M NaOH for another 30 minutes. This treatment was repeated, the exchanger was filtered and washed with distilled water until its pH was 7.0. The exchanger was poured into a column and equilibrated with 10 mM sodium phosphate, pH 8.0.

The dialysed immunoglobulin solution was passed through the DEAE-52 column in 10 mM sodium phosphate, pH 8.0 at 4°C. The non-binding proteins were collected, precipitated with ammonium sulphate (291 g/litre) at 4°C and then recovered by centrifugation at 10,000 g

at 4°C for 10 minutes. The precipitate was dissolved in the minimum quantity of 10 mM Tris/HCl, pH 7.0 containing 0.15 M NaCl and dialysed against the same buffer at 4°C overnight. The purified IgG solution was stored at -20°C.

D. Estimation of Antibody Titre

The potency of the anti-lipoprotein lipase antibody was estimated in vitro and in vivo. Antibody titres were compared to the potency of control serum or control IgG which had been obtained from a non-immunised sheep.

In vitro Potency

The in vitro potency of antisera and IgG preparations was determined by the immunoprecipitation of a fixed source of lipoprotein lipase with increasing dilutions of antibody.

Antibody preparations were diluted with 0.9% saline over a range 1:10 to 1:1,000. Adipose lipoprotein lipase (0.03 μmol FFA/μl/hour) purified by Heparin-Sepharose CL-6B affinity chromatography (see above) was used as the enzyme source. 40 μl enzyme source was incubated with 160 μl 0.9% saline and 200 μl of the various antibody dilutions at 4°C for 30 minutes. 20 μl donkey anti-sheep IgG antiserum (Scottish Antibody Production Unit, Carluke, Scotland) was added and incubated at 4°C for a further 30 minutes. After centrifuging at 1,500 g at 4°C for 30 minutes, the supernatant was assayed for Lpl activity (see Appendix for results).

In vivo Potency

The in vivo potency of antisera and IgG preparations was determined by the increase in plasma VLDL concentration caused by the inhibition of the functional Lpl. Plasma VLDL concentration was measured by the turbidimetric assay developed by Griffin and Whitehead (1982).

The turbidimetric assay reagent was 20 mM Tris/HCl, pH 7.4 containing 150 mM NaCl, 150 mM $MgCl_2$ and 0.025% (w/v) heparin.

A plasma sample was taken prior to the injection of 2 ml antibody source and again 60 minutes after the injection. 200 μl plasma was added to 2.0 ml turbidimetric assay reagent, vortexed thoroughly and left at room temperature for 20 minutes. The optical density was then read at 540 nm.

The in vivo potency of the antibody source was given by the increase in plasma VLDL over the hour period compared with the change caused by the control serum (see Appendix for results).

XII. METABOLISM OF LABELLED LIPOPROTEINS IN VIVO

The uptake of labelled palmitic acid presented in different forms was investigated in vivo. This was achieved by introducing labelled unesterified fatty acid bound to albumin, labelled portomicrons, and labelled VLDL into recipient hens and tracing the fate of the label in a variety of tissues 15 minutes later.

A. Preparation of Labelled Lipoproteins

^{14}C-Palmitic Acid Bound to Albumin

Using a gentle stream of nitrogen the carrier toluene was removed from 10 μCi palmitic acid-1-^{14}C, specific activity 58 mCi/mmol, (Amersham International plc) which was then resuspended in 500 μl ethanol. An excess of 0.01 M NaOH was added and allowed to stand at room temperature for 30 minutes to produce sodium ^{14}C-palmitate. The mixture was evaporated under nitrogen and then redissolved in 100 μl distilled water using a water-bath at 60°C. After cooling, 1.25 ml fresh hen serum was added.

The majority of the sodium ^{14}C-palmitate became associated with serum albumin (Bragdon and Gordon, 1958).

^{3}H-Portomicrons

Portomicrons, containing labelled palmitic acid, were synthesised in vivo by a donor bird and then isolated by ultracentrifugation.

Food had been withheld for 18 hours from the donor bird, a 36 week old Isabrown hen. Solvent containing 300 μCi palmitic acid-[9-10-^{3}H], specific activity 46 Ci/mmol, (Amersham International plc) was evaporated down under nitrogen and redissolved in 650 μl ethanol and 5 ml distilled water. The [^{3}H]-palmitic acid solution was introduced directly into the empty crop via a long stemmed intubation funnel, followed by rinsings of 5 ml olive oil, 3 g feed and 10 ml water.

The in vivo lipoprotein lipase activity was inhibited to aid the accumulation of nascent portomicrons by the administration of 2.5 ml anti-lipoprotein lipase IgG after 60 and 90 minutes. The donor bird was injected with a lethal dose of sodium pentobarbitone 120 minutes after intubation and blood collected from the jugular vein. Plasma was recovered by centrifugation at 1,000 g at 4°C for 10 minutes. The plasma in 16.5 ml centrifuge tubes, was overlaid with at least 4 ml 0.9% saline and centrifuged at 110,000 g at 10°C for 1 hour in a swing-out bucket rotor. The top 1 cm was removed from each centifuge tube, combined and overlaid again with 0.9% saline and recentrifuged as before. The isolated portomicrons were removed in the minimum volume and stored at 4°C to be used within 24 hours.

A 50 μl aliquot of the ^{3}H-portomicrons was added to 4.5 ml scintillation fluid (Optiphase "X", Fisons, UK) and counted. It was

estimated that 44 x 10^6 dpm in the form ^{3}H-portomicrons had been isolated by the above method.

^{14}C Very Low Density Lipoproteins

VLDL, containing labelled palmitic acid, were synthesised *in vivo* by a donor hen and then isolated by ultracentrifugation.

200 μCi ^{14}C-palmitic acid was bound to serum albumin, as described above. The donor bird, a 36 week old Isabrown hen which had been allowed access to food, was injected with 2.5 ml anti-lipoprotein lipase IgG in one wing and the ^{14}C-palmitate bound to albumin in the other. A further 2.5 ml anti-lipoprotein lipase IgG was administered after 30 minutes. After 60 minutes, plasma was obtained as in the preparation of ^{3}H-portomicrons. Plasma, in 16.5 ml centrifuge tubes, was overlaid with at least 4 ml 0.9% saline and spun at 110,000 g at 10°C for 1 hour. The portomicrons and overlay were removed and discarded while the remaining tube contents had their overlay adjusted with 0.9% saline. The ^{14}C-VLDL were isolated by re-centrifuging at 110,000 g at 10°C for 20 hours, removed and resuspended in the minimum volume of 0.9% saline. The isolated ^{14}C-VLDL were stored at 4°C and used within 24 hours.

A 25 μl aliquot of the ^{14}C-VLDL was added to 4.5 ml scintillation fluid and counted. It was estimated that 11 x 10^6 dpm in the form of ^{14}C-VLDL had been isolated by the above method.

B. Characterisation of Labelled Lipoproteins

The *in vivo* generation of labelled ^{3}H-portomicrons, as well as ^{14}C-VLDL, utilised the anti-lipoprotein lipase IgG to inhibit the donor hens' Lpl activity and, hence, prevent the hepatic uptake of ^{3}H-portomicrons and the subsequent synthesis and secretion of ^{3}H-VLDL. The

distribution of label between the lipoprotein classes was established by gel filtration, whilst the distribution of the label between the lipoprotein lipids was determined by thin-layer chromatography (TLC).

Distribution of Label Between Lipoproteins

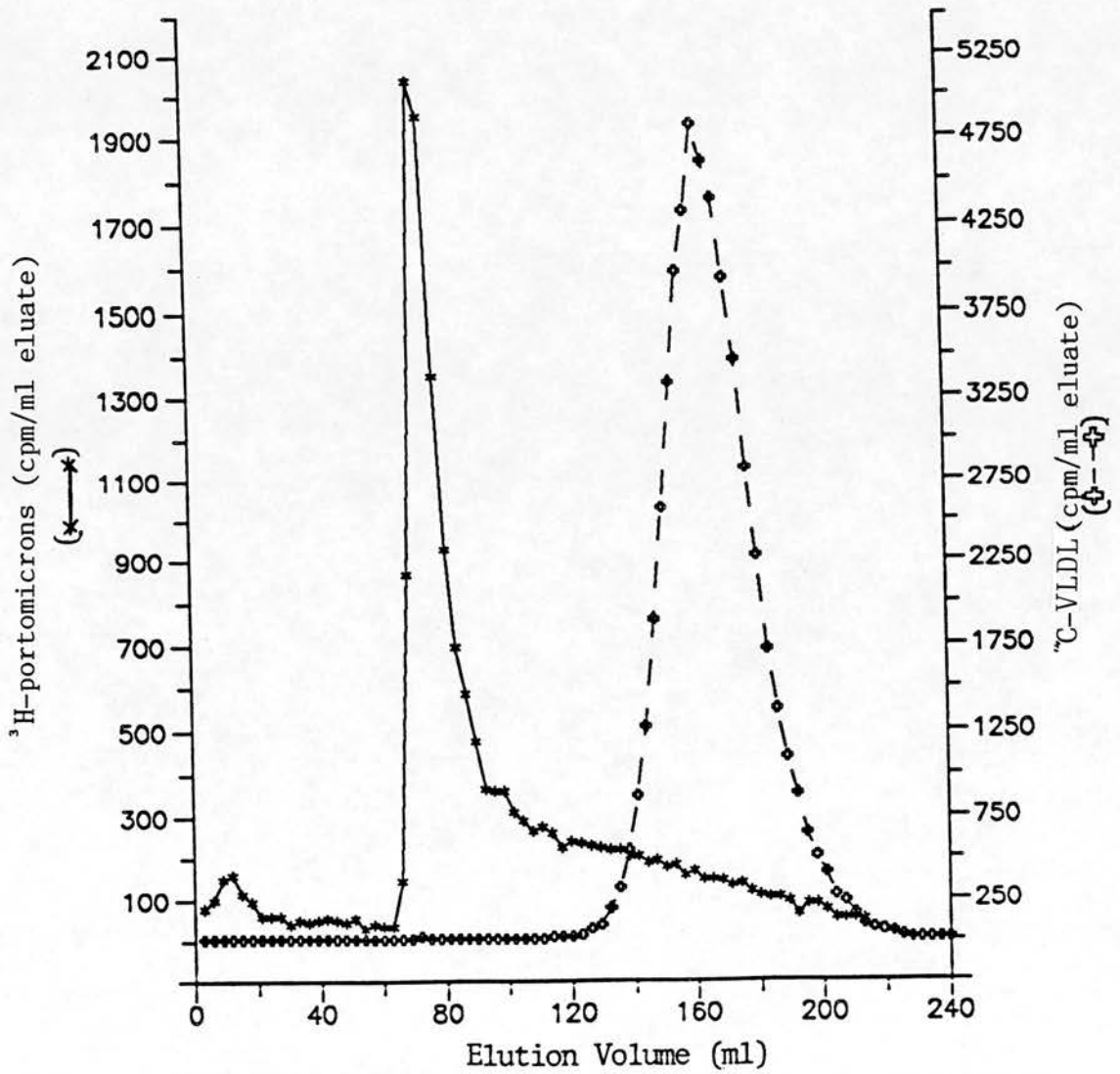
Following the ultracentrifugal isolation of the ^{3}H-porto-microns, a 200 μl aliquot was subjected to gel filtration on an agarose column (see above) and 3 ml fractions collected. 500 μl/fraction was added to 4.5 ml scintillation fluid (Optiphase "X", Fisons, UK) and counted. The isolated ^{14}C-VLDL were treated in an identical manner.

It can be seen from Fig. 2.1 that the use of anti-lipoprotein lipase IgG and the ultracentrifugal isolation procedure ensured the purity of the labelled lipoprotein fractions. There appeared to be no ^{3}H-VLDL present, but rather that tritium was solely associated with the portomicrons.

Distribution of Label Within the Lipoprotein

Lipid was extracted from 100 μl ^{3}H-portomicrons and ^{14}C-VLDL separately, by the method of Bligh and Dyer (1959) (see above). The extracted lipid was resuspended in 0.5 ml chloroform and loaded onto a TLC plate (200 x 200 x 0.5 mm) the solid phase being silica gel-H (type 60) (Merck, Darmstadt, West Germany). The plate was developed using heptane:diethyl ether:acetic acid (160:40:2 v/v). The position of the lipids was identified by iodine vapour and then extracted from the silica by washing twice with chloroform and centrifuging at 500 g for 5 minutes. Phospholipid was extracted using chloroform:methanol:water (5:5:1 v/v/v). The solvent was evaporated at 40°C under nitrogen and the lipid resuspended in 5 ml scintillation fluid (Optiscint "T", Fisons, UK) and counted.

Fig. 2.1: Demonstration of the purity of ^{3}H-portomicrons and ^{14}C-VLDL by gel filtration on an agarose-column.



EXPERIMENTAL DETAILS

Portomicrons or VLDL were isolated by centrifugation from a donor bird either fed 300 µCi ^{3}H-palmitic acid or injected with 200 µCi ^{14}C-palmitic acid, respectively. A 200 µl aliquot of the isolated portomicrons was fractionated by column chromatography on a Bio-Gel A-150 column (43x2.6cm), as described in Chapter 2. 3ml fractions were collected and 500 µl/fraction was added to 4.5ml scintillation fluid and counted. The isolated ^{14}C-VLDL were treated in an identical manner.

The distribution of the label between the different lipid classes in ^{3}H-portomicrons and ^{14}C-VLDL is shown in Table 2.1. As expected, over 90% of the labelled palmitic acid was incorporated in the triglyceride in both lipoproteins.

Table 2.1: The distribution of the label between the lipid classes in portomicrons and VLDL

	%	
LIPID	^{3}H-Portomicrons	^{14}C-VLDL
Phospholipid	1.36	7.40
Cholesteryl ester	0.46	0.44
Triglyceride	95.48	90.60
Fatty Acid	1.69	0.52
Cholesterol	0.64	1.03

C. The Fate of Labelled Lipoproteins In vivo

Labelled Tissues

The labelled lipoproteins were injected via the brachial vein into the recipient hens, which were known to be laying regularly. The birds were replaced in individual cages for 15 minutes. A 5 ml blood sample was taken, placed in a tube with 50 μl 0.2 M EDTA, pH 7.4 and centrifuged at 1,000 g at 4°C for 10 minutes to obtain plasma. A lethal dose of sodium pentobarbitone was administered and the tissues of interest were removed immediately, rinsed in chilled 0.9% saline, blotted dry, weighed and frozen in liquid nitrogen. The tissues were stored at -80°C.

Liquid Scintillation Counting of Labelled Lipids

Labelled lipids were extracted from the tissues and plasma using a modification of the method described by Folch et al. (1957) (see above).

The extracted lipids were transferred to scintillation mini-vials with 4 ml chloroform:methanol (19:1 v/v) and dried in a vacuum oven at 45°C. 4.75 ml scintillation fluid (Optiscint "T", Fisons, UK) was added to the mini-vials, thoroughly vortexed and left in the dark for 48 hours.

A double quench curve (^{3}H and ^{14}C) was established in the LKB 1216 Rack Beta II scintillation counter (LKB Instruments Ltd, Surrey) using a mixture of β-carotene and carbon tetrachloride as quenching agents. The chemiluminescence was also recorded and this was subtracted from the total counts.

Estimation of Tissue Blood Volume

To distinguish between labelled lipid uptake by tissue cells and the presence of labelled lipid in plasma within the tissue, the volume of plasma within tissues was determined using radioiodinated human serum albumin (Bragdon and Gordon, 1958).

12.5 μCi ^{125}I-albumin, (Amersham International plc) was mixed with the labelled lipoproteins before being injected into the recipient birds. Approximately 1 gram of tissue, weighed to 3 decimal places, and 50 μl plasma was placed in tubes and counted on the LKB 1272 Clinigamma Automatic gamma counter (LKB Instruments Ltd, Surrey).

XIII. PLASMA OESTRADIOL DETERMINATION

Plasma oestradiol concentrations were determined using a double-antibody radioimmunoassay (RIA) developed by Webb et al. (1985) following an initial purification similar to that described by Peterson and Common (1972); Johnson and Tienhoven (1980); Hagan at al. (1984) and Leszczynski et al. (1984) to remove the large amount of lipid present in laying hen plasma.

A. Reagent Purity

To minimise the interference of residual solvent impurities in the RIA, high quality solvents were employed. Ethanol and ethyl acetate (BDH, Poole) were "Aristar" grade. All other solvents used were glass redistilled high performance liquid chromatography grade solvents supplied by Rathburn Chemicals Ltd (Walkerburn, Scotland).

B. Plasma Sample Collection

All the plasma samples (2.5 ml) for an experiment were obtained between 8.30 am and 10.30 am on a single day and stored at -20°C. Only birds in the middle of an egg clutch and known to have laid an egg on the day of plasma collection and on the preceding day and following day were included in the oestradiol determination. These precautions enabled samples to be obtained from birds at similar stages within their oestrus cycle and, thus, reduce the intra-treatment variation.

C. ^{3}H-Oestradiol-17β Purification

Oestradiol losses during the sample processing prior to RIA were determined for all plasma samples by determining the recovery of tritiated oestradiol of high specific activity used as an internal standard. As labelled steroids are known to undergo considerable decomposition on storage, the labelled oestrogen was purified before each series of oestradiol determination.

Preswollen Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden) in ethyl acetate:methanol (9:1) was packed in a glass pipette plugged with glass wool to a bed volume of 7 ml and equilibrated with the same solvent system. The column was allowed to drain and 150 μCi [2,4,6,7,16,17-^{3}H] oestradiol-17 β, (specific activity 156C/mmol; Amersham International plc) was applied and eluted with the same solvent system. Fractions of 0.5 ml were collected, diluted to 1.5 ml with ethanol and 15 μl aliquots taken for counting. Peak fractions were pooled and dried down under a gentle stream of nitrogen. The purified ^{3}H-oestradiol was resuspended in a small volume of ethanol and stored at -20°C.

Thin-layer chromatography was performed to verify the purification of the ^{3}H-oestradiol. An aliquot of ^{3}H-oestradiol, mixed with unlabelled oestradiol-17β (Sigma), was separated on a 0.5 x 200 x 200 mm plate of Silica gel-H (type 60) (Merck, Darmstadt, West Germany) using chloroform:acetone (70:30 v/v) as developing solvent. The position of oestradiol-17 β on the plate was identified with iodine vapour. Essentially all of the label loaded onto the plate was associated with this band, as determined by liquid scintillation following the extraction of the silica with ethanol.

D. Plasma Sample Processing

Plasma samples were processed prior to the RIA based on the method of Peterson and Common (1972); Johnson and Tienhoven (1980); Hagan et al. (1984), and Leszcynski et al. (1983), though a different solvent system was employed. Diethyl ether extracted all the lipid (including oestradiol) from the plasma and chromatography on Sephadex LH-20 was then used to separate the oestradiol from the lipids and other steroids.

Oestradiol Extraction

The purified ^{3}H-oestradiol was diluted with ethanol to approximately 5,000 cpm/200 μl. To 0.5 ml aliquots of plasma samples 200 μl ^{3}H-oestradiol was added and mixed thoroughly and then refrozen.

After thawing, the plasma samples with internal standards were vortexed with 5 ml diethyl ether. After centrifugation at 500 g at 0°C for 10 minutes, the upper organic phase was removed with a pasteur pipette and the lower phase was re-extracted with a further 5 ml diethyl ether. The combined organic layers were dried down at 35°C under a gentle stream of nitrogen.

The extracted samples were stored in stoppered test tubes at -20°C.

Oestradiol Partial Purification

Sephadex LH-20 was swollen and stored in the solvent system:- isooctane:toluene:methanol (62:20:18 v/v). The gel slurry was pipetted into 5 ml glass syringe barrels (1 cm diameter) which had tightly fitting Whatman GF/D glass filter paper discs inserted at the bottom. Further gel was added to give a settled bed volume of 3 ml and this was washed with 10 ml solvent system. Another glass filter paper disc was placed on top of the gel and the column was washed with a further 10 ml solvent system and the column was allowed to drain dry.

The extracted oestradiol samples were resuspended in 200 μl solvent system at 35°C and placed on the glass filter paper disc with a pasteur pipette. The column was eluted with 3.0 ml solvent system and allowed to drain dry, the eluate being discarded. Oestradiol which was eluted with a further 10 ml solvent system was collected in glass stoppered tubes and dried down using a Buchler Vortex evaporator (Gallenkamp).

The partially purified oestradiol samples were stored at -20°C until ready for RIA.

LH-20 columns were used only once since high and inconsistent blank values with repeated use have been reported (Peterson and Common, 1972).

E. Oestradiol Radioimmunoassay

The radioimmunoassay employed to determine oestradiol concentrations was that established at the AFRC Institute of Animal Physiology and Genetics Research, Edinburgh and modified from that described by Webb et al. (1985).

The diluent (PBS-gel) used throughout the assay was phosphate buffered saline containing gelatin (0.05 M Na phosphate, 0.15 M NaCl; pH 7.5 containing 0.01% thiomersal and 0.1% gelatin).

A stock solution of oestradiol-17β (100 pg/ml) dissolved in PBS-gel was used for constructing a calibration curve over the range 0-48 pg/tube. The oestradiol antiserum used in the assay had been raised against 17 β-oestradiol-11β-succinyl-bovine serum albumin in rabbits. The labelled hormone was iodinated 17 β-oestradiol-11α-succinyl-tyrosinemethylester.

Three "total" counts tubes, containing labelled oestradiol only, gave a measure of unbound labelled ^{125}I-oestradiol added. The unspecific binding of the labelled hormone was given by three "blank" tubes containing all reagents except the anti-oestradiol antiserum. Six "zero standard" tubes, containing all reagents except the unlabelled oestradiol, represented 100% binding of the antiserum to the labelled hormone.

Assay Protocol

The oestradiol samples were resuspended in 1.5 ml PBS-gel buffer and 150 μl was dispensed, in duplicate, with the volume made up to 500 μl with diluent. Nine standards (0.5-48.0 pg/tube), each in triplicate, were dispensed and made up to 500 μl with diluent. The three "blank" and six "zero standard" tubes contained 500 μl diluent. 100 μl ^{125}I-oestradiol (containing about 20,000 counts per 100 seconds) was dispensed into all the tubes. 200 μl oestradiol antiserum, diluted with PBS-gel buffer (1:40,000 v/v) was dispensed into all of the tubes, except for the totals and blanks. The three blanks were given 200 μl diluent. The tubes were thoroughly shaken and incubated at room temperature for 3 hours.

100 μl donkey anti-rabbit IgG serum (Scottish Antibody Production Unit, Carluke, Scotland), diluted to 1:40 (v/v) with PBS-gel buffer, containing 0.1 M EDTA, was added to all of the tubes, except for the totals. 100 μl normal rabbit serum, diluted to 1:400 (v/v) with PBS-gel buffer, containing 0.1 M EDTA, was dispensed to all the tubes, except the totals. The tubes were thoroughly vortexed and incubated at 4°C overnight.

Except for the totals, 1 ml ice-cold PBS-gel buffer was added to all the tubes and centrifuged at 800 g at 4°C for 30 minutes. The supernatant was immediately decanted and any remaining liquid droplets on the lip of the tubes were aspirated.

The precipitated antigen-antibody complex at the bottom of the tube was counted for 100 seconds on a gamma counter (LKB 1271 Riagamma). The data was recorded on a Serius microcomputer before being transferred to a Prime 550 mainframe computer for processing.

Validation of Oestradiol Radioimmunoassay

The oestradiol radioimmunoassay itself has been well characterised and validated for use with plasma samples from sheep (Webb et al. 1985). However, the preliminary sample processing by diethyl ether extraction followed by Sephadex LH-20 treatment could introduce large unsystematic errors. Hence, the overall oestradiol determination needed to be validated for its reproducibility, precision and sensitivity.

Reproducibility: Plasma was pooled from several hens and ten 0.5 ml aliquots were processed, as described above, prior to oestradiol determination. The standard deviation expressed as a percentage of the mean gave an estimate of the intra-assay coefficient of variation. All samples from a single experiment were always arranged in the same RIA, hence, an estimate of inter-assay variation was unnecessary.

Precision: Nine 0.5 ml aliquots of pooled plasma were dispensed and 3 volumes (in triplicate) of oestradiol-17β, dissolved in ethanol, was added. These plasma samples were processed as for the unknowns and the correlation between the amount of oestradiol added and the amount determined gave an indication of the precision of the assay.

Sensitivity: The sensitivity of the assay, defined as the concentration of hormone required to give a response two standard deviation units higher than the zero standard response, was calculated.

Solvent Interference: Despite using high quality solvents, an estimate of the unspecific binding of hormone due to solvent impurities was necessary. Four 0.5 ml distilled water aliquots were processed as described for the plasma samples. The amount of "oestradiol" determined was called the "solvent blank".

Oestradiol Recovery

The percentage ^{3}H-oestradiol recovery was determined to account for oestradiol losses during the plasma sample processing prior to the RIA.

After the processed plasma samples were resuspended in 1.5 ml PBS-gel buffer, 500 μl was transferred to scintillation mini-vials with 4.5 ml scintillation fluid (Optiphase "X", Fisons Ltd, UK). The samples were counted in the LKB 1216 Rackbeta II scintillation counter together with 200 μl stock ^{3}H-oestradiol.

Treatment of Radioimmunoassay Data

The RIA data were computed using the ABRO RIA programme package which is based on the method of Rodbard and Lewald (1970). The computer programme was designed for a double-antibody RIA which linearised the sigmoid standard curve by iterative least squares regression following logit:log transformation. For each pair of unknown duplicates a mean oestrodiol value was calculated together with the 95% confidence limits.

The mean solvent blank and individual percentage ^{3}H-oestradiol recoveries were taken into account in the calculation of the plasma oestradiol concentration.

XIV. EXPERIMENTAL DATA ANALYSIS

All experimental data were stored on a Prime 550 mainframe computer and manipulated using the Sheffield Prime Editor. The data were subjected to statistical analysis using the Minitab Statistics Package (Version 5) supported on the Prime 550 mainframe. The data analysis methods employed in the study included paired t-tests,

Duncan's multiple range test (Duncan, 1955), least significance differences, one- and two-way analysis of variance, linear correlation, and both linear and quadratic regression. The method of analysis used for a particular set of data is given in the appropriate Experimental Protocol section.

CHAPTER 3

THE EFFECT OF DIETARY FAT ON EGG SIZE - A STUDY AT THE GROSS LEVEL

I. INTRODUCTION

The mechanism by which dietary fat increases egg weight is not known, though it has generally been assumed to act via an increase in yolk lipid deposition and, hence, yolk size. However, the evidence for such an assumption is not convincing and there have been reports of dietary fat increasing egg weight without affecting yolk size (see Chapter 1 for a more detailed discussion). In the study of the effect of dietary fat on egg weight it was necessary to establish whether the increase in weight was due to yolk or another of the egg components (i.e. albumen or shell).

A review of the literature reveals that the presence and the magnitude of egg weight responses to different fat types are variable (see Chapter 1 and Table 1.2). The most consistent responses appear to be obtained with unsaturated vegetable oils high in linoleic acid. Maize oil, at an inclusion level of 55 g/kg, was chosen as the dietary fat to establish a satisfactory experimental model for investigating the effect of "dietary fat" on egg weight.

The response of egg weight to dietary fat is 3-5% of the whole egg weight at best and requires careful experimental design to detect. Preliminary examination of egg weights laid by a group of hens over a month showed that within-bird variation was much less than between-hen variation over the period, with coefficients of variation of 2.9% and 4.7% respectively. It was decided to utilise this lower within-bird component of egg weight variation to improve the sensitivity of the experimental design. The effect of dietary fat upon egg weight was not determined by simply comparing the mean egg weight at the end of a period on different dietary treatments. Instead, egg weight for

individual hens was determined both before and after the commencement of dietary treatment. Calculating the change in egg weight for the individual hens gave a more sensitive test for the effect of the dietary treatment.

II. EXPERIMENT 1: THE EFFECT OF DIETARY MAIZE OIL ON EGG WEIGHT AND EGG COMPONENTS

i) Objectives

A preliminary experiment was designed to confirm that an increase in egg weight could be detected following maize oil supplementation. A further objective was to establish whether any increase in egg weight was as a result of increased yolk weight and if this was due to a greater lipid deposition.

ii) Experimental Protocol

Eighty-four Isabrown hens were reared to 45 weeks of age as described previously (Chapter 2). Egg weights were recorded over a 7 day period and the birds were ranked into 7 groups of 12 birds on the basis of their pre-experimental egg weights so as to minimize within-group variation. Each group of 12 birds was split into two sub-groups and then fed the experimental diets as below.

All birds were fed the low-fat diet for weeks 46 and 47. During this period egg, yolk, albumen and shell weights were determined daily. Half the birds were then fed the high-fat diet from weeks 48 to 57, whilst the other half remained on the low-fat diet. Egg, yolk, albumen and shell weights were determined during weeks 56 and 57. Towards the end of this period yolk and albumen were separated from 3 eggs for each bird and pooled. The dry matter contents and yolk lipid were determined on these pooled samples, as described in Chapter 2.

All of the birds were fed the low-fat diet during weeks 58 and 59.

Egg production and whole egg weights were recorded daily throughout the experimental period. Shell-less, yolk-less and double yolked eggs were included in the egg production data, but their weights were not recorded.

Diets: A low- and a high-fat diet were formulated to be isonitrogenous and isoenergetic, composed of practical ingredients (Table 3.1) and were fed ad libitum in mash form. The additional fat content of the high-fat diet was provided by 55 g maize oil/kg. The ether-extractable lipid contents of the low- and high-fat diets were 18 g/kg and 75 g/kg, respectively.

Data Analysis: The change in egg weight over the experimental period was calculated for each bird from the pre- and post-treatment means. All other measurements were compared in the same manner, except for yolk and albumen dry matter and yolk lipid for which post-treatment values only were recorded.

The data were subjected to one-way analysis of variance according to Steel and Torrie (1980).

iii) Results

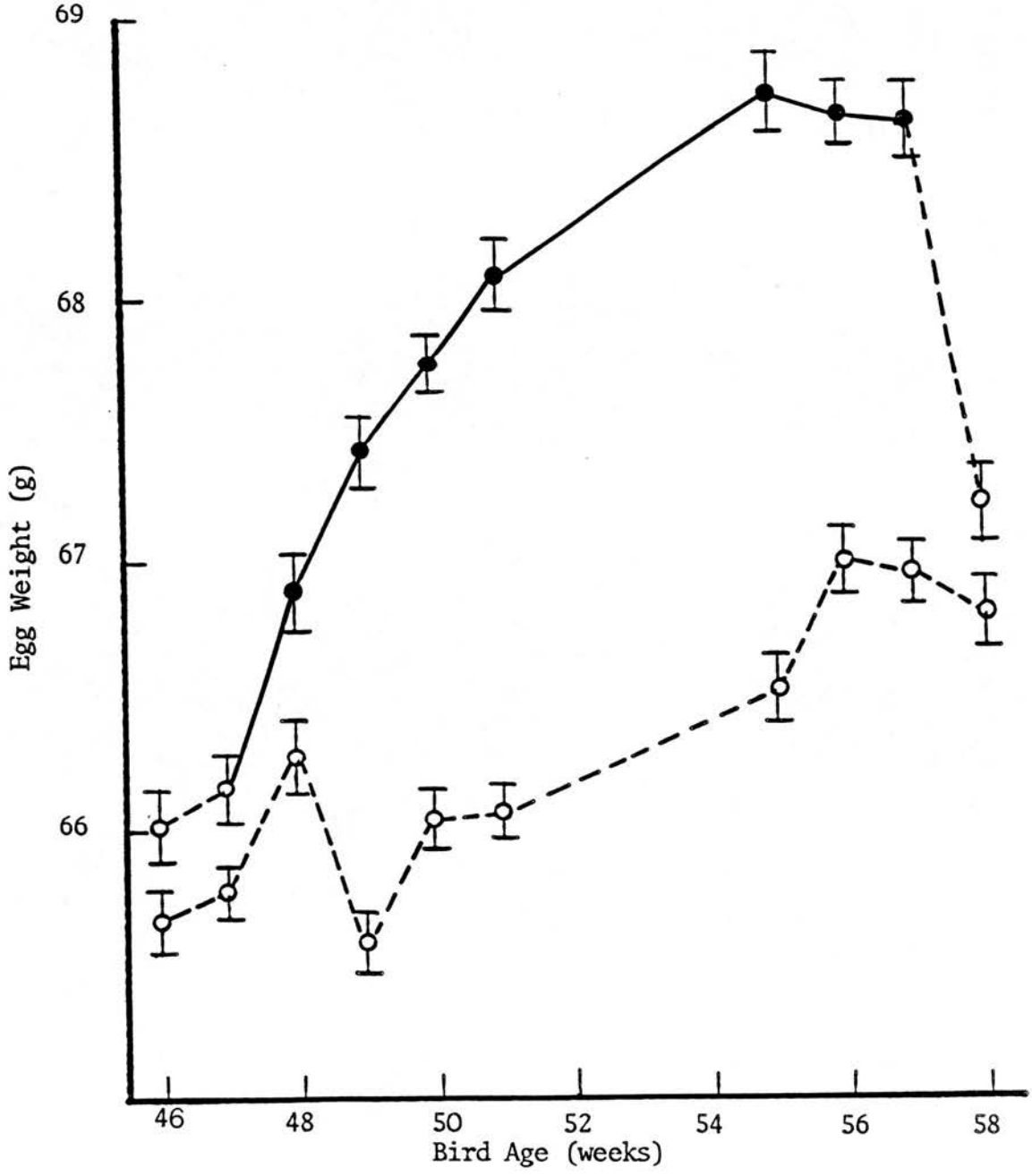
The effect of the dietary treatments on egg weight with relation to age is shown in Fig. 3.1. As expected, egg size increased with the age of the birds on both diets. The high-fat diet elicited a larger increase in egg weight, but after week 50 the eggs from birds on both treatments appeared to increase in a parallel fashion. When birds previously fed the high-fat diet were given the low-fat diet a considerable reduction in egg size was noted.

Table 3.1: Composition of experimental diets

	Low-Fat	High-Fat
INGREDIENT (g/kg)		
Wheat	765.0	150.0
Wheatfeed	-	420.0
Soyabean Meal	25.0	110.0
Herring Meal	65.0	45.0
Meat and Bone Meal	30.0	30.0
Barley	-	46.0
Dried Grass Meal	-	45.0
Limestone Flour	65.0	67.0
Dicalcium Phosphate	22.0	23.0
DL-Methionine	-	1.0
Protein Supplement[a]	20.0	-
Sodium Chloride	3.0	3.0
Vitamin Supplement[b]	2.5	2.5
Mineral Supplement[c]	2.5	2.5
Maize Oil	-	55.0
Determined Analyses (g/kg)		
Crude Protein	166.0	164.0
Ether Extractives	18.0	75.0
Calcium	36.0	35.0
Phosphorus	7.0	8.0
Calculated Analyses (per kg)		
Linoleic Acid (g)	6.5	37.9
Metabolisable Energy (MJ)	11.42	11.42

a FPD-950 (95% protein), I.C.I. England
b Supplies per kg diet: Vit A 10,000 I.U; Vit D 2,000 I.U; Vit E 25 I.U; Menadione 2 mg; Riboflavin 4 mg; Nicotinic Acid 28 mg and Pantothenic Acid 10 mg
c Supplies per kg diet: Zinc 50 mg; Copper 3.6 mg; Iodine 0.4 mg; Iron 80 mg; Manganese 100 mg and Selenium 0.15 mg.

Fig3.1:The response in egg weight (±SEM) to a low-fat diet (○) and a high-fat diet (●) in hens aged between 46 and 58 weeks old.



The changes in egg, yolk, albumen and shell weight are shown in Table 3.2, together with the changes in the proportions of these egg components expressed as a percentage of the whole egg weight. The maize oil supplemented diet significantly increased the change in egg ($P<0.05$) and albumen ($P<0.01$) weight. Neither yolk nor shell weight were affected by the dietary treatment. As a proportion of the whole egg weight, yolk and albumen slightly increased over the 10 week experimental period in birds fed the low-fat diet, whilst shell weight was slightly reduced. Dietary fat significantly increased the proportion of albumen ($P<0.05$) in the egg compared to birds fed the low-fat diet with a concomitant decrease in the proportion of yolk ($P<0.05$). The relative amount of shell was unaffected.

There was a decline in the number of eggs laid by birds on both dietary treatments with age (Table 3.3) and this was not significantly affected by fat supplementation. Though maize oil increased mean egg weight, there was no significant difference in the mass of eggs produced by birds on either treatment.

Yolk lipid and dry matter values for the yolk and albumen are shown in Table 3.4. Birds fed the high-fat diet produced yolks containing a higher proportion of lipid (g/g of yolk), this increase being small but significant ($P<0.05$). As would be expected, concomitant with an increase in yolk lipid content there was a significant increase in the proportion of dry matter (w/w) in the yolk. The proportion of dry matter in the albumen was significantly ($P<0.05$) increased by the feeding of the maize oil supplemented diet.

Table 3.2: The effect of dietary treatment upon the change in the weight (g+SEM) of the egg and the internal components and upon the change in the relative proportions of the internal components weights (% of the whole egg weight + SEM) between the weeks 46 and 58

	DIETARY TREATMENT		Significance of Treatment[a]
	Low-Fat	High-Fat	
Changes in weight (g)			
Whole egg	+1.290±0.35[b]	+2.549±0.29	*
Yolk	+0.520±0.10	+0.545±0.15	NS
Albumen	+0.906±0.29	+2.249±0.17	**
Shell	-0.200±0.29	-0.250±0.20	NS
Changes in Relative Proportions (% of whole egg weight)			
Yolk	+0.232±0.18	-0.220±0.14	NS
Albumen	+0.157±0.35	+1.140±0.21	*
Shell	-0.492±0.37	-0.945±0.28	NS

[a] NS, Not significant; *P<0.05; **P<0.025; ***, P<0.01, **** P<0.005
[b] Values are the mean of 7 groups of 6 birds.

EXPERIMENTAL DETAILS

All birds were fed the low-fat diet for weeks 46 and 47. During this period egg,yolk,albumen, and shell weights were determined daily. Half the birds were then fed the high-fat diet from weeks 48 to 57 whilst the other half remained on the low-fat diet. Egg,yolk,albumen and shell weights were again determined during weeks 56 and 57.

Table 3.3: The effect of dietary treatment upon the change in daily egg production (+SEM) between the weeks 46 and 58

	DIETARY TREATMENT		Significance of Treatment[a]
	Low-Fat	High-Fat	
Changes in Egg Production			
Daily egg number (%)	-3.911±1.30[b]	-6.292±0.50	NS
Daily egg mass (g)	-2.010±0.38	-2.170±0.39	NS

[a,b] See Table 3.2.

EXPERIMENTAL DETAILS

All birds were fed the low-fat diet for weeks 46 an 47 . During this period egg weights and numbers were determined daily. Half the birds were then fed the high-fat diet from weeks 48 to 57 whilst the other half remained on the low-fat diet. Egg weights and numbers were again determined daily during weeks 56 and 57.

Table 3.4: The effect of dietary treatment upon the composition (+SEM) of yolk and albumen

	DIETARY TREATMENT Low-Fat	High-Fat	Significance of Treatment[a]
% Yolk lipid (g/g of yolk)	35.184±0.160b	35.671±0.150	*
% Yolk dry matter (g/g)	52.326±0.085	52.647±0.065	*
% Albumen dry matter (g/g)	13.034±0.093	13.406±0.140	*

[a,b] See Table 3.2.

EXPERIMENTAL DETAILS

All birds were fed the low-fat diet for weeks 46 and 47. Half the birds were then fed the high-fat diet from weeks 48 to 57 whilst the other half remained on the low-fat diet. During week 57 yolk and albumen were separated from 3 eggs for each bird and pooled. The dry matter contents were determined by freeze-drying and the yolk lipid contents were determined gravimetrically by a 2-step Folch extraction method ,as described in Chapter 2.

iv) Conclusion

A diet containing 55 g maize oil/kg was shown to increase egg weight when compared to a low-fat, isonitrogenous and isoenergetic diet. The response was large over the initial two weeks, after which the effect was maintained but not improved upon. The extra egg weight due to dietary fat was lost within 7 days of returning the birds to the low-fat diet. Since no difference in yolk weight could be detected, it is concluded that the increase in egg weight due to the maize oil supplemented diet is brought about almost entirely through increased albumen secretion. The lipid content of the yolk (g/g of yolk) was slightly greater in the fat-fed birds and albumen dry matter was also increased.

III. EXPERIMENT 2: THE EFFECT OF HEN AGE AND DIETARY MAIZE OIL ON EGG WEIGHT AND EGG COMPONENTS - A COMPARISON

i) Objectives

The findings of the initial experiment that dietary fat increased egg weight not via yolk, but by increased albumen weight were surprising and unsupported by any previous published reports. Thus, it was necessary to determine if these results could be confirmed in a second experiment. In addition, it was of interest to compare the relative proportions of the yolk, albumen and shell weight in eggs which had attained similar weights either by age or dietary fat.

ii) Experimental Protocol

Sixty Isabrown hens were reared to 19 weeks of age as described previously (Chapter 2). Egg weights were recorded daily during week 20 and the birds were ranked into five groups of twelve birds on the basis

of egg weight so as to minimise within-group variation. Each group of birds was split into two sub-groups and then fed the experimental diets ad libitum, as below. The diets used were the same as those in EXPERIMENT 1 and their compositions are given in Table 3.1.

All birds were fed the low-fat diet during weeks 21 and 22 with the egg, yolk, albumen and shell weights being determined during this period. Half the birds were transferred to the high-fat diet from week 23 onwards whilst the other half remained on the low-fat diet. Egg, yolk, albumen and shell weights were again determined daily during weeks 31 and 32. When eggs from birds fed the low-fat diet were estimated to have attained a mean weight similar to those that had been laid by birds on the high-fat diet during weeks 31 and 32, the egg, yolk, albumen and shell weights were again determined daily for two weeks. This occurred when the birds were $42\frac{1}{2}$-$43\frac{1}{2}$ weeks old.

Egg production and egg weights were recorded throughout the experiment.

Albumen was separated and pooled from three eggs for each bird during week 43 and freeze-dried to determine its dry matter content. The protein content of this lyophilised material was determined by the Kjeldhal method, as described in Chapter 2.

The data were subjected to one-way analysis of variance according to Steele and Torrie (1980).

iii) Results

The response of egg weight to both changes in hen age and dietary treatment is shown in Fig. 3.2. Regardless of diet, egg size increased as the birds aged, although the rate of this increase diminished as the experiment progressed. The high-fat diet initially

Fig. 3.2: Daily mean egg weights in groups of hens from 21 to 44 weeks of age fed low fat (○) and high fat (●) diets.

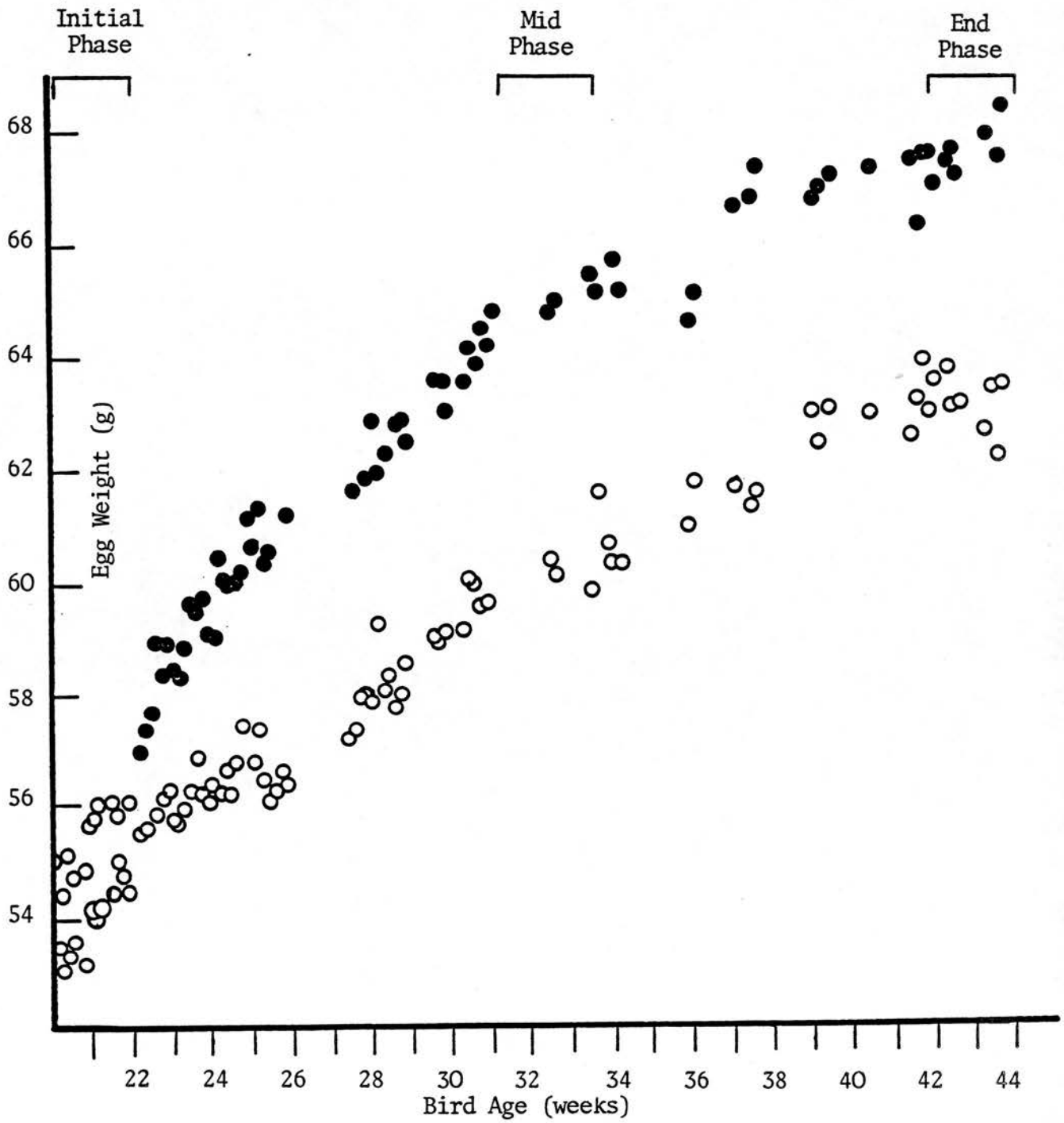
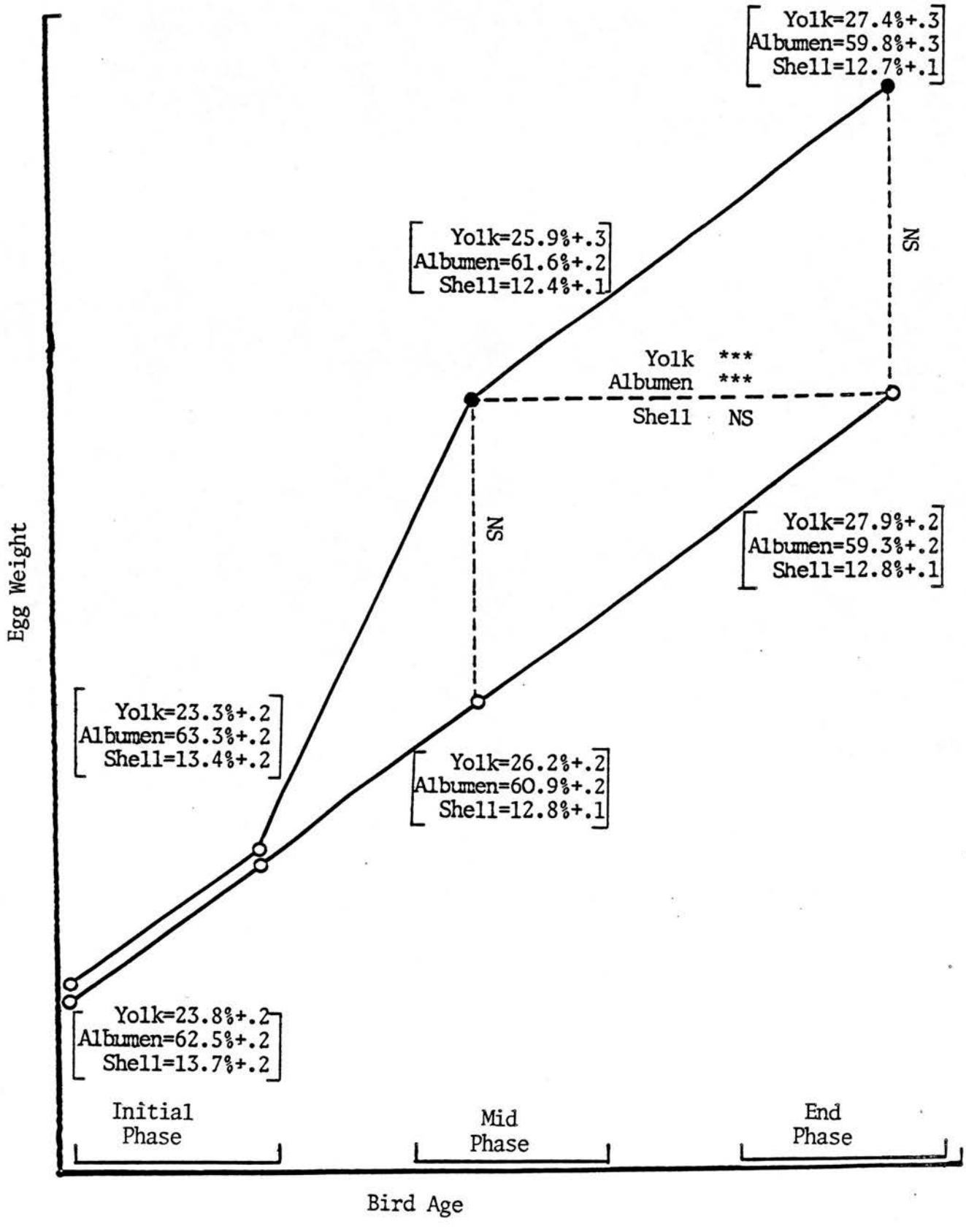


Fig3.3:A schematic diagram to illustrate the effect of age and either a low-fat diet (O) or a high-fat diet (●) upon the weights of the egg components,expressed as apercentage of the total egg weight(±SEM).

NS,not significant
***,P<0.001



elicited a large response in egg weight, though after about 3 weeks, egg size on both diets increased in a parallel fashion. This observation was confirmed when the changes in egg weight between the fortnights 21/22 and 31/32 weeks old were compared with those between the fortnights 31/32 and $42\frac{1}{2}/43\frac{1}{2}$ weeks old (Table 3.5). Initially, the first ten weeks of feeding the high-fat diet caused a significant ($P<0.05$) increase in egg weight of 2.5 g, and though this difference was maintained, there was no significant additional effect of dietary fat on egg weight during the subsequent eleven weeks. Likewise, the high-fat diet caused a significant increase in albumen ($P<0.005$) and yolk ($P<0.025$) weight only in the initial phase. No effect on shell weight due to the dietary treatment was noted at any time during the experiment.

The composition of the egg changed during the experimental period (Fig. 3.3). As the hens aged, the yolk formed a larger proportion of the egg at the expense of the proportion of albumen. Dietary fat did not cause a significant difference in the change of these proportions (Table 3.5) nor the proportions themselves (Fig. 3.3). However, if the proportions of the internal components of eggs from birds fed the low-fat diet during weeks $42\frac{1}{2}-43\frac{1}{2}$ (end-phase) are compared with those eggs of the same weight laid by hens fed the high-fat diet during weeks 31-32, (mid-phase) the different effects of age and diet can be seen (Fig. 3.3). Compared with eggs which had attained the same weight through age, dietary fat caused a significant increase ($P<0.001$) in the proportion of albumen, at the expense of yolk which was significantly lower ($P<0.001$).

Table 3.5: The change in the weight of egg components (g+SEM) and the relative proportions of the internal components' weights (% of the whole egg weight +SEM) in response to ageing and dietary fat

Age of Hens	23 to 32 weeks			32 to 43 weeks		
	DIETARY TREATMENT		Significance of	DIETARY TREATMENT		Significance of
	Low-Fat	High-Fat	Treatment[a]	Low-Fat	High-Fat	Treatment[a]
Changes in weight (g)						
Whole egg	+5.680±0.28[b]	+8.153±0.34	****	+3.496±0.28	+3.685±0.63	NS
Yolk	+2.825±0.15	+3.571±0.20	**	+1.969±0.16	+1.986±0.26	NS
Albumen	+2.596±0.18	+4.125±0.22	****	+1.117±0.19	+1.031±0.27	NS
Shell	+0.259±0.15	+0.457±0.10	NS	+0.409±0.11	+0.668±0.14	NS
Changes in relative proportions (% of whole egg weight)						
Yolk	+2.479±0.22	+2.625±0.28	NS	+1.662±0.18	+1.514±0.17	NS
Albumen	−1.597±0.12	−1.629±0.32	NS	−1.596±0.26	−1.827±0.24	NS
Shell	−0.881±0.28	−0.994±0.11	NS	−0.066±0.16	+0.312±0.09	NS

[a] See Table 3.2
[b] Values are means of 5 groups of 6 birds.

EXPERIMENTAL DETAILS

All the birds were fed the low-fat diet for weeks 21 and 22 with the egg,yolk,albumen and shell weights being determined daily during this period. Half the birds were transferrred to the high-fat diet from week 23 onwards whilst the other half remained on the low-fat diet. Egg,yolk,albumen and shell weights were determined daily during weeks 31 and 32 and again during weeks 42 ½ and 43 ½.

Though feeding the high-fat diet did not significantly affect the dry matter content of the albumen (w/w) or the protein content of this dry matter (w/w) (Table 3.6), dietary fat significantly ($P<0.05$) increased the amount of albumen dry matter and protein laid per egg.

iv) Conclusion

Egg weight was increased by over 2.5 g in birds fed a maize oil supplemented diet compared to a low-fat isonitrogenous and isoenergetic diet. This increase was rapid, but it was shown that feeding fat for a further 11 weeks had no additional effect upon the initial increase. Both yolk and albumen contributed to the increase in egg weight. Eggs from birds fed the maize oil diet contained a higher proportion of albumen and less yolk than similar sized eggs laid by birds on the low-fat diet 11 weeks older. This would indicate that dietary fat increased egg weight in a manner different to the increase in egg weight associated with the age of the hen.

IV. EXPERIMENT 3: CONFIRMATION OF THE INFLUENCE OF HEN AGE ON THE RESPONSE OF EGG WEIGHT AND EGG COMPONENTS TO DIETARY MAIZE OIL

i) Objectives

Two studies had been performed to investigate which of the internal components of the egg are affected by the feeding of a maize oil supplemented diet and had produced conflicting results (see above). One trial had shown that dietary fat only affected albumen weight (EXPERIMENT 1) whilst the other trial showed that both yolk and albumen weight were affected (EXPERIMENT 2). In the first trial using older birds (45 weeks old) yolk size only increased by approximately

Table 3.6: The effect of dietary treatment upon the composition (+SEM) of albumen

	DIETARY TREATMENT Low-Fat	High-Fat	Significance of Treatment[a]
% Dry matter (g/g of albumen)	12.616±0.19[b]	12.811±0.12	NS
Total albumen dry matter (g/egg)	4.727±0.15	5.167±0.11	*
% Protein (g/g of dry albumen)	83.255±F0.33	83.344±0.37	NS
Total albumen protein (g/egg)	3.937±0.13	4.307±0.10	*

[a] See Table 3.2
[b] See Table 3.5.

EXPERIMENTAL DETAILS

All the birds were fed the low-fat diet for weeks 21 and 22. Half the birds were transferrred to the high-fat diet from week 23 onwards whilst the other half remained on the low-fat diet. Albumen was separated from the yolk and pooled from 3 eggs for each bird during week 43 and freeze-dried to determine its dry matter content. The protein content of this lyophilised material was determined by the Kjeldhal method,as described in Chapter 2.

0.5 g over the 10 week experimental period due to age. However, with the younger birds (22 weeks old) in the second trial yolks increased due to age by approximately 3.0 g in 8 weeks, and by a further 2.0 g over the subsequent 11 weeks. This suggested that dietary fat could only influence yolk size in young hens when the rate of increase in yolk size was high, after which the effect of dietary fat was either absent or undetectable.

To test the above hypothesis a third experiment was designed to confirm that maize oil supplementation affected egg weight via albumen weight alone in older birds.

ii) Experimental Protocol

The thirty hens fed the low-fat diet (Table 3.1) in EXPERIMENT 2 remained on the same diet until 48 weeks old. The birds were divided into two groups of 15 birds, each group having approximately the same overall mean egg weight. Egg, yolk, albumen and shell weights were recorded daily during weeks 48 and 49. Half the birds were then transferred to the high-fat diet (Table 3.1), whilst the other half remained on the low-fat diet. The whole egg and internal component weights were again recorded daily during weeks 57 and 58.

The thirty hens fed the high-fat diet in EXPERIMENT 2 were treated in the reverse manner to that above.

iii) Results

Unfortunately the experimental size prevented many effects from being detected at the 5% significance level, though the general responses obtained were similar to those in previous experiments (Table 3.7 and 3.8).

Birds transferrred from the low-fat diet to the maize oil

Table 3.7: The effect of dietary treatment upon the change in the weight (g+SEM) of the egg and its internal components between the weeks 49 and 57

	DIETARY TREATMENT		Significance of Treatment[a]
	Low-Fat	High-Fat	
Changes in weight (g)			
Whole egg	+1.039±0.62[b]	+2.184±0.52	NS
Yolk	+0.356±0.23	+0.620±0.30	NS
Albumen	+0.020±0.40	+1.270±0.47	*
Shell	+0.660±0.32	+0.285±0.25	NS

[a] See Table 3.2
[b] Values are the mean of 15 birds.

EXPERIMENTAL DETAILS

All the birds were fed the the low-fat diet for weeks 21 to 49. Half the birds were transferred to the high-fat diet from week 50 onwards whist the other half remained on the low-fat diet. Egg,yolk,albumen and shell weights were determined daily during weeks 48 and 49 and then again during weeks 57 and 58.

Table 3.8: The effect of transferring birds from the high-fat diet to the low-fat diet upon the change in the weight (g±SEM) of the egg and its internal components between the weeks 49 and 57

	DIETARY TREATMENT		Significance of
	Low-Fat	High Fat	Treatment[a]
Changes in weight (g)			
Whole egg	-0.56±0.67[b]	+1.38±0.61	NS
Yolk	-0.01±0.30	+0.09±0.21	NS
Albumen	-0.89±0.50	+1.24±0.56	***
Shell	+0.34±0.18	+0.05±0.31	NS

[a] See Table 3.2
[b] Values are the mean of 15 birds.

EXPERIMENTAL DETAILS

All the birds were fed the the high-fat diet for weeks 23 to 49. Half the birds were transferred to the low-fat diet from week 50 onwards whist the other half remained on the high-fat diet. Egg, yolk, albumen and shell weights were determined daily during weeks 48 and 49 and then again during weeks 57 and 58.

supplemented diet laid eggs which were heavier by over 1.0 g. The increase in egg weight caused by dietary fat appeared to be entirely due to the significant increase ($P<0.05$) in albumen weight. Yolk weights increased only slightly over the 10 week experimental period and dietary fat did not appear to affect this increase. No effect of dietary treatment upon shell weight was noted.

A large reduction in egg weight was observed when birds formerly fed the maize oil supplemented diet were transferred to the low-fat diet. Withdrawal of dietary fat did not appear to affect the yolk or shell weight, but there was a significant ($P<0.01$) reduction in albumen weight.

iv) Conclusion

A maize oil supplemented diet increased egg weight by over 1.0 g in 50 week old hens by increasing the weight of albumen secreted without affecting yolk or shell weight. Conversely, withdrawal of dietary fat caused egg weight to be reduced by 2.0 g, and this was due to a decrease in albumen secretion, without affecting yolk or shell weight. Therefore, it would appear that in older birds the response in egg weight due to dietary fat is via an effect upon albumen weight alone.

V. DISCUSSION

A series of small-scale nutritional trials was conducted to establish a satisfactory experimental model for investigating the effect of dietary fat upon egg weight. The two diets employed were isonitrogenous and isoenergetic, with the high-fat diet containing 55 g maize oil/kg. Though the diets were formulated to be adequate in all

known nutrients, the linoleic acid content of the low-fat diet, 6.5 g/kg, was slightly below the recommended requirement of 9 g/kg (Whitehead, 1981). However, since the birds had been reared on a linoleic acid-adequate diet it can be assumed that their adipose tissue reserves would ensure no deleterious effect of the low linoleic acid diet. The difficulty of inducing linoleic acid deficiency in adult hens was discussed in Chapter 1.

Compared to a low-fat diet, an isonitrogenous and isoenergetic diet containing 55 g maize oil/kg increased egg weight by 2.5 g in young birds and 1.3 g in older birds. These increases in egg weight were within the range similar to those found by numerous other workers (e.g. Balnave, 1967 and 1971a; Whitehead, 1981; Hoyle and Garlich, 1987), but not consistent with the conclusion of Morris (1985) who suggested that dietary manipulation could enhance egg weight by a maximum of 1.0 g. The egg weight response was large over the initial 2 weeks after which the effect was maintained but not improved upon, an observation also noted by Edwards and Morris (1967). Egg weight was reduced within 7 days of transferring birds from a high- to a low-fat diet. The rapid increases and decreases in egg weight due to the presence or absence of dietary fat have been suggested to be a method for the short term manipulation of egg weight (Balnave, 1987).

By comparing the effect of dietary treatments upon the change in the weights of the egg and its internal components over a fixed period rather than the actual weights at a fixed time, a more sensitive method of data analysis was developed enabling small responses in yolk and albumen weights to be detected. It was generally believed that dietary fat increased egg weight via yolk weight by increasing the

amount of yolk lipid deposited (see Chapter 1). Yolk weight has been reported to increase with dietary fat supplements by many workers (Jensen et al., 1958; Jensen and Shutze, 1963; Balnave, 1970; Sell et al., 1987), whilst albumen weight remained unaffected (Sell et al., 1987). However, dietary fat has also been noted to increase egg weight without affecting yolk size (Guenter et al., 1971; Karunajeewa and Tham, 1987). The present study demonstrated an age-dependent effect of dietary fat upon egg weight. Dietary fat caused a large increase in egg weight in young hens by increasing both yolk and albumen weight. The smaller egg weight increase observed in older hens was associated with albumen weight only, yolk weight being unaffected. It is possible that the conflicting published reports on the effect of dietary fat upon the internal components of the egg could be accounted for by the use of less sensitive data analysis methods (see above) and differences in the age of the hens used.

Explanations for the age-dependent effect of dietary fat upon internal components are not obvious. In assuming an asymptotic relationship between egg weight and age, Weatherup and Foster (1980) presupposed that the egg has a fixed theoretical maximum weight which is attained more slowly as it is approached with time. Jenkins and Taylor (1960) also suggested that yolk and albumen have theoretical maximum weights which the bird approaches at first rapidly and then more slowly. The results in this study indicate that dietary fat can only affect yolk weight in young birds when the rate of increase in yolk weight due to age is high. It may be that the theoretical maximum yolk weight had nearly been reached in the trials using older hens (>45 weeks old) and, hence, dietary fat could not further increase yolk

deposition. Dietary fat increased albumen weight in both young and old hens. This may be because albumen does not attain its maximum weight until the hen is considerably older, or perhaps the stimulation of albumen secretion caused by dietary fat can circumvent the restricting physiological processes responsible for the maximum attainable value.

The maize oil-supplemented diet also caused changes in the composition of the yolk and albumen. Yolk lipid (g/g of yolk) was slightly increased by dietary fat in the present study, though this has not been shown in other studies (Combs and Helbacka, 1960; Balnave, 1967). This may be due to differences in the methods used to determine the total yolk lipid contents. Most workers used a standard extraction method according to Folch et al. (1957), but the present study employed a two-step Folch extraction method (Chapter 2). This latter method was demonstrated to have a coefficient of variation of <0.5% (data not shown) which is substantially better than the single Folch extraction value of 9.0% (Fletcher et al., 1984). The more accurate method of analysis may be necessary to detect the small increases in yolk lipid caused by dietary fat. The increase in yolk lipid due to dietary fat supports the supposition that yolk weight is increased via greater yolk lipid deposition, though it should be noted that this increase was only slight.

Balnave (1971b) reported that maize oil increased the proportion of dry matter in albumen (w/w), this observation being confirmed in one trial of the present study but not in the other. However, in both trials, eggs from birds fed the maize oil supplemented diet contained approximately 0.4 g more albumen protein. Hence, it would appear that dietary fat increases the amount of albumen protein synthesised by the hen.

The nutritional trials in the present study provided information about the effect of dietary fat on different egg components. The specific mechanisms involved in these effects were subsequently investigated using a biochemical approach (Chapter 4).

CHAPTER 4

THE EFFECT OF DIETARY FAT ON EGG SIZE - A STUDY AT THE BIOCHEMICAL LEVEL

Chapter 3 described how the effect of dietary fat on egg weight was investigated at the gross level by means of a series of sequential nutritional trials. This chapter describes experiments designed to investigate the biochemical mechanisms responsible for the findings of the previous chapter. Though dietary fat has the ability to increase both yolk and albumen weight, the former is only affected in young birds. Hence, the possible theories describing the biochemical mechanisms by which dietary fat increases yolk deposition and albumen secretion appear to be independant and will be dealt with separately.

SECTION A. THE EFFECT OF DIETARY FAT ON YOLK PRECURSORS

I. INTRODUCTION

It was shown in the previous chapter that dietary fat affected yolk deposition in an age-dependant manner, the yolk size increasing in 24 week old hens, but not in hens older than 45 weeks. The increased yolk weight was accompanied by a slightly greater lipid content (w/w). Dietary fat, in the form of portomicrons, can not be deposited directly into the oocyte due to their large size, but rather must be firstly remodelled by the liver into the smaller VLDL (see Griffin et al., 1984; and Griffin and Perry, 1985). It was therefore postulated that dietary fat could spare the yolk precursors (VLDL) from being partially degraded before they were incorporated into the oocyte.

In the laying hen, under the influence of oestrogen, the liver secretes VLDL which are very different from immature hen VLDL, an

apparent adaptation to yolk formation. Compared to the immature hen VLDL, the laying hen VLDL concentration is about 20-fold greater (Yu et al., 1976), VLDL are smaller with a broader density distribution (Kudzama et al., 1979) and have different lipid compositions (Chapman et al, 1977) The unusually small size of laying hen VLDL allows its passage through the granulosa basal lamina surrounding the developing oocyte (Griffin and Perry, 1985). Another property of laying hen VLDL to aid yolk deposition is their limited susceptibility to hydrolysis by the enzyme lipoprotein lipase (Lpl).

As VLDL travel from their site of synthesis, they are subjected to partial hydrolysis in the extra-hepatic tissues by Lpl, and it was postulated that dietary fat may decrease this partial degradation of VLDL. Dietary fat, in the form of portomicrons, is also a substrate for Lpl. Chylomicrons, the mammalian equivalent of portomicrons, contain more Lpl-activator apoprotein than VLDL (Cryer, 1981). It is thus reasonable to assume that the affinity of Lpl for portomicrons will be much higher than for laying hen VLDL. Indeed, this assumption is supported by the greater susceptibility of portomicrons to Lpl than VLDL *in vitro* (Griffin *et al.*, 1982). The plasma concentration of portomicrons increases with fat-feeding (Griffin *et al.*, 1982), a condition aided by the relatively low tissue activity of Lpl in laying hens. It is possible that the hydrolysis of VLDL is reduced by the presence of more portomicrons for which Lpl has a higher affinity resulting in more and larger VLDL reaching the oocyte and being incorporated into the yolk. As VLDL enter the yolk via receptor-mediated endocytosis (Griffin *et al.*, 1984), an increase in VLDL size may be of greater importance.

In order to test the above hypothesis, the effect of dietary fat on plasma lipoproteins was investigated. In addition, the response of the hen's Lpl activity to dietary fat was investigated. The enzyme Lpl is thought to be synthesised at a site distinct from its functional location which is attached to the luminal surface of the capillary endothelial cells by heparin-like glycosaminoglycan chains. The functional Lpl can be displaced into the circulation by intravenous injection of heparin (see Cryer, 1981), and its activity measured in the plasma. This post-heparin lipase activity (PHLA) gives the sum of all the tissues' Lpl activities and indicates the functional Lpl status of the bird.

Chapter 3 showed that the effect of dietary fat upon yolk size was age-dependant - an increase in yolk size was only detectable in young birds. Could the postulated hypothesis that dietary fat spares the degradation of the yolk precursors, VLDL, accommodate this age-dependant characteristic? Age causes Lpl activity to decline in adipose tissue (Angervall, 1960 and Chlouverakis, 1962) and in aortal tissue (Zemplenyi, 1962), while Bradows and Campbell (1972) reported the PHLA of 86 year old human subjects to be less than half that of 28 year olds. A decline in the hen's lipase activity with age could explain why dietary fat exerts less of an effect on yolk size in older birds. By diverting the degradative action of Lpl from VLDL to portomicrons dietary fat can increase yolk deposition only as long as the Lpl activity is high enough to influence the amount of VLDL reaching the oocyte.

To provide evidence in support of the hypothesis that dietary fat enhances yolk deposition by decreased VLDL degradation and to

test whether a reduction of this phenomenum with age is responsible for the lack of effect that dietary fat exerts on yolk size in old birds the following were investigated:

(i) the effect of dietary fat upon triglyceride-rich plasma lipoproteins and PHLA and

(ii) the effect of age upon PHLA.

II. EXPERIMENTAL PROTOCOL

Twenty Isabrown hens, aged 25 weeks, were fed either the low-fat or the high-fat diet (see Table 3.1 for composition) ad libitum for 4 weeks. 1.5 ml blood was collected from 5 birds on the low-fat diet and combined before plasma being recovered. Portomicrons and VLDL were isolated from the plasma by ultracentrifugation (see Chapter 2) and resuspended in 5 ml elution buffer. The lipoproteins were then subjected to gel filtration and the presence of the lipoproteins in the fractions was determined by absorbance at 280 nm. The procedure was repeated in an identical manner using 5 birds fed the high-fat diet.

For 6 birds on each dietary treatment portomicrons were isolated from 3.75 ml plasma by ultracentrifugation (see Chapter 2). Lipid was extracted from the portomicron fraction and from 500 μl aliquots of the remaining centrifuge tube, referred to as the "VLDL fraction"*, by the method according to Bligh and Dyer (1959) and the triglyceride content of the extracted lipid was determined.

* Though the "VLDL fraction" consists of VLDL, LDL and HDL, over 93% of the triglyceride is present in the VLDL (calculated from Chapman, 1980).

A 2 ml post-heparin plasma sample was removed from the wing-vein of all 20 birds 2 minutes after an injection of 500 I.U. heparin/kg body weight. The total PHLA and the hepatic lipase activity was determined as described in Chapter 2.

The PHLA was determined, as above, in Isabrown hens aged between 20 and 84 weeks which were all maintained on a standard layers diet (25 g/kg fat).

Details of experimental methods are described in Chapter 2.

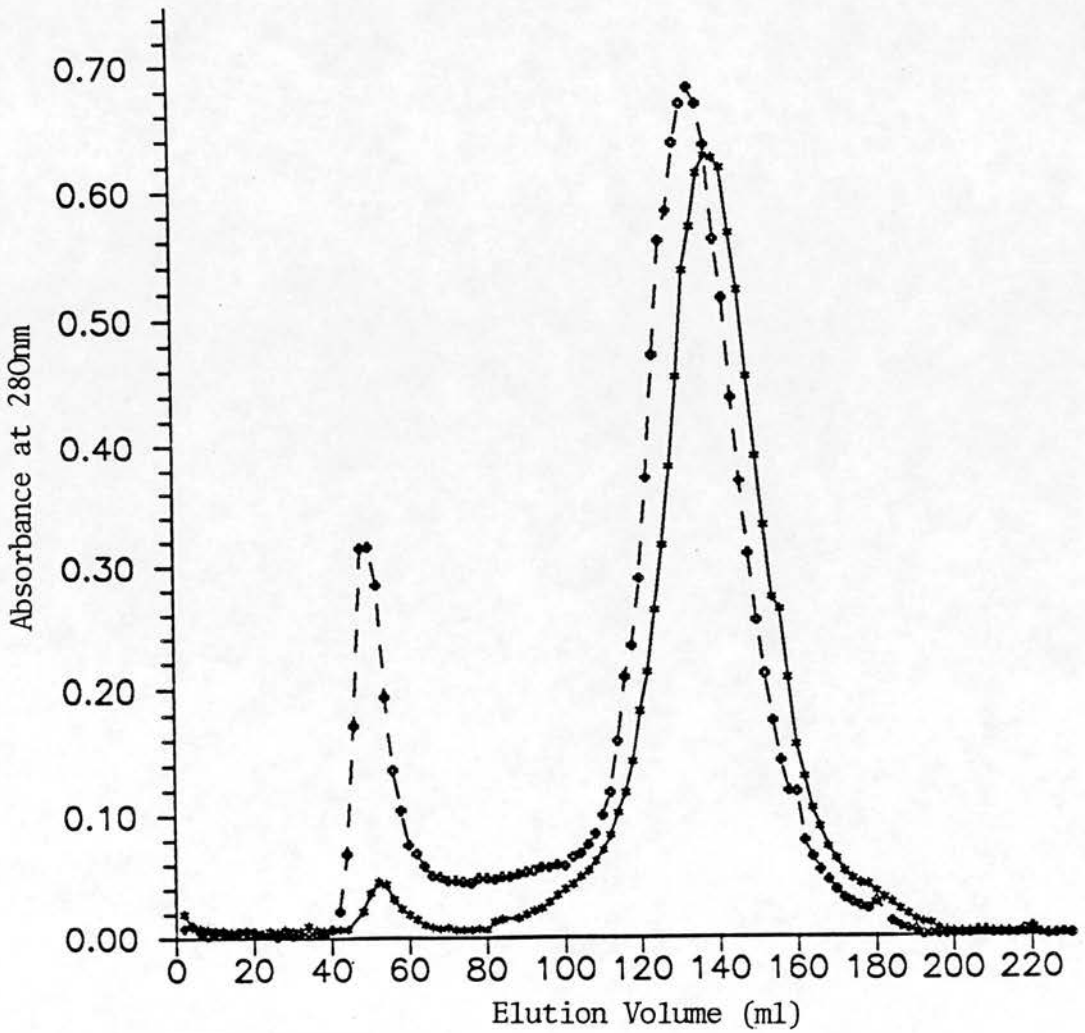
III. RESULTS

(i) The effect of dietary fat upon triglyceride-rich plasma lipoproteins and post-heparin lipase activity (PHLA)

Portomicrons and VLDL from birds fed the low- and high-fat diets were chromatographed on a column containing Agarose A-150 m (Fig. 4.1). The larger portomicron fraction was eluted in the first peak, the second peak consisting of VLDL showing a broad size distribution. As expected, dietary fat caused an accumulation of portomicrons in the plasma, whilst in birds fed the low-fat diet only a minor peak corresponding to the portomicron fraction was detected. The VLDL peaks were of a similar magnitude on both treatments, though it appeared that dietary fat marginally advanced the elution of the VLDL peak, indicating an increase in particle size.

Dietary fat appeared to increase VLDL triglyceride concentration, but due to the large within-treatment variance the difference failed to attain statistical significance (Table 4.1). Triglyceride in the circulation was mainly in the VLDL fraction on both treatments, but the ratio of VLDL:portomicron triglyceride in the fat-fed birds was a quarter that found in birds on the low-fat diet ($P<0.02$).

Fig 4.1:Agarose-column chromatography of triglyceride-rich lipoproteins from the plasma of hens fed on a low-fat diet (✳) and a high-fat diet (✜).



EXPERIMENTAL DETAILS

Triglyceride-rich lipoproteins were isolated by the centrifugation of the combined plasma (5.5ml) from 5 hens fed a low-fat diet or a high-fat diet for 4 weeks and fractionated by chromatography on a Bio-Gel A-150m column (43x2.6cm),as described in Chapter 2. The presence of the lipoproteins in the fractions collected (2ml) was determined by absorbance at 280nm.

Table 4.1: The effect of dietary fat on plasma portomicron and VLDL triglyceride concentration (+ SEM) in laying hen plasma

	Low-Fat Diet (n=6)	High-Fat Det (n=6)	P[1]
Portomicron-triglyceride (μmol/ml)	0.232±0.106	1.404±0.340	<0.002
VLDL-triglyceride (μmol/ml)	11.649 ±2.911	16.455±2.377	NS
Ratio VLDL:Portomicron triglyceride	47.5 ±13.7	11.4 ±2.4	<0.002

[1] Means separated by Student's t-test

EXPERIMENTAL DETAILS

IsaBrown hens, aged 25 weeks, were fed either the low-fat or the high-fat diet for 4 weeks. Portomicrons were isolated by centrifugation from 3.75ml plasma samples from 6 birds on each diet. Lipid was extracted from the portomicron fraction and from 500 μl aliquots of the remaining fraction, referred to as the "VLDL fraction" (see above). The triglyceride content of the extracted lipid was determined as described in Chapter 2.

Table 4.2: The effect of dietary fat upon post-heparin lipase activity (+ SEM)

Post-heparin Lpl activity	Low-Fat Diet (n=10)	High-Fat Diet (n=10)	P[1]
μmol/hr/ml plasma	5.55±1.808	16.135±1.808	<0.001
μmol/hr/bird[2]	651 ±206	1945 ±200	<0.001

[1] Means separated by Student's t-test

[2] Lipase activity calculated per bird based on the assumption of a packed-cell volume of 25% and blood volume of 80 ml/kg body weight (Freeman, 1984)

EXPERIMENTAL DETAILS

IsaBrown hens, aged 25 weeks, were fed either a low- or high-fat diet (see Table 3.1 for composition) for 4 weeks. A 2ml post-heparin plasma sample was taken 2 minutes after an injection of 500 I.U. heparin/kg body weight from the wing vein. The PHLA was assessed as described in Chapter 2.

The hepatic lipase activity present in the post-heparin plasma, as determined in the supernatant after incubation with anti-lipoprotein lipase antiserum, was less than 1% of the total PHLA regardless of dietary treatment (results not shown). Therefore, the post-heparin Lpl was considered to be the PHLA. Dietary treatment was shown to significantly ($P<0.001$) affect PHLA (Table 4.2). Compared with the low-fat diet, birds fed the diet containing 55 g/kg maize oil had almost three times the functional lipase activity which could be displaced into the circulation with an injection of heparin.

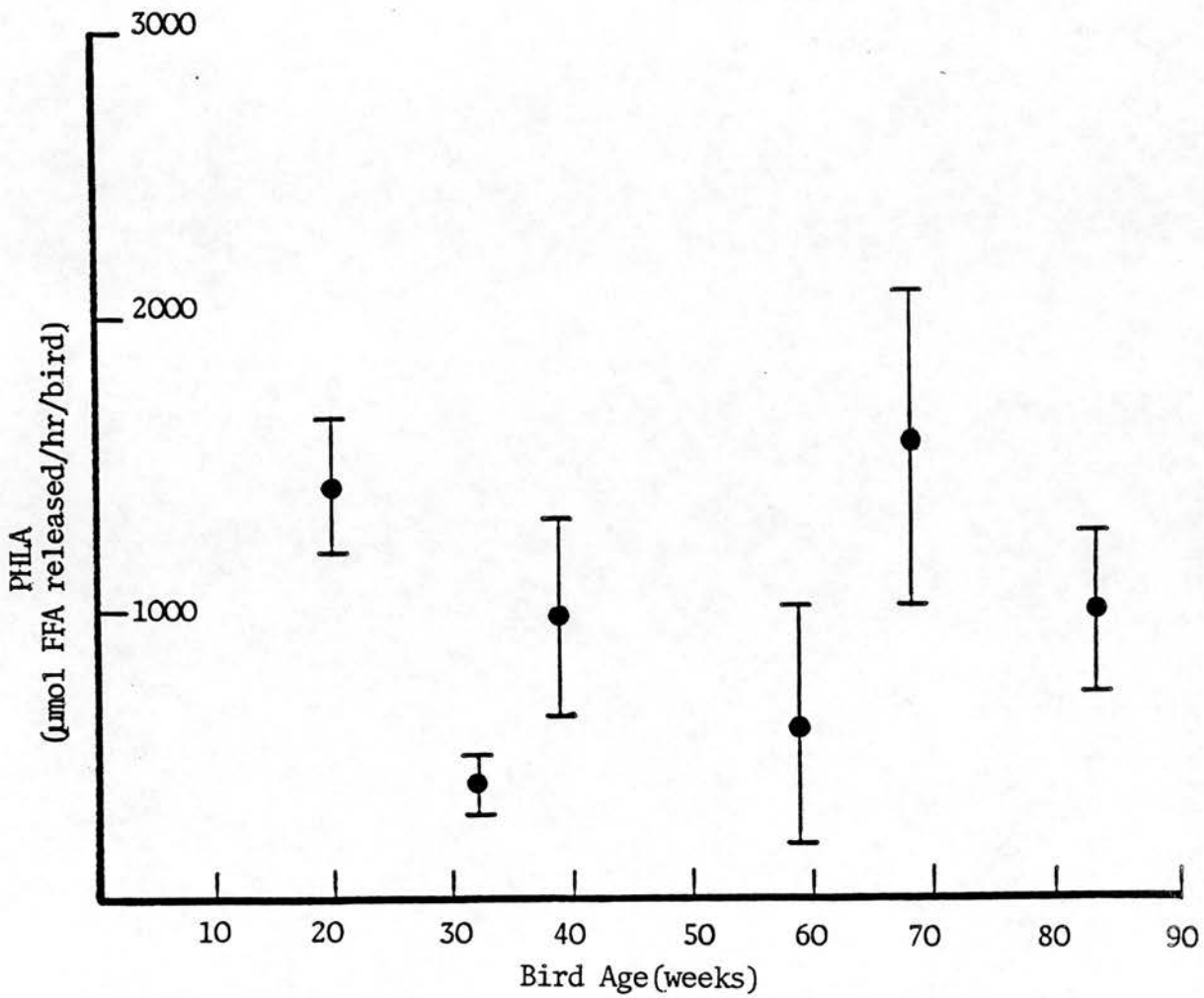
(ii) The effect of age upon post-heparin lipase activity (PHLA)

The birds used to investigate how PHLA changed with age were from six groups ranging from 20 to 84 weeks old. As above, hepatic lipase activity was negligible (<1.5% total PHLA in any age group, results not shown) and the post-heparin Lpl was considered to be the PHLA. There was great variation exhibited in PHLA within any age group and the coefficient of variation ranged from 42-161%. With such a degree of variation it was not surprising that no trend could be detected between PHLA and age (Fig. 4.2). There appeared to be no correlation between the rate of lay or the position in the clutch sequence of a particular hen and her PHLA which might have explained the variation in a bird's lipase activity.

IV. DISCUSSION

An attempt was made to obtain evidence relevant to the hypothesis that dietary fat might decrease VLDL degradation, thus resulting in more plasma VLDL triglyceride and larger VLDL particles.

Fig4.2:The relationship between hen age and the post-heparin lipase activity (PHLA).The values at each age are the means (±SD) of five hens.



EXPERIMENTAL DETAILS

A 2ml post-heparin plasma sample was taken 2 minutes after an injection of 500 I.U. heparin/kg body weight from the wing-vein of IsaBrown hens aged between 20 and 80 weeks. All birds were fed a standard layers diet (25g fat/kg). The PHLA was assessed as described in Chapter 2.

However, careful deliberation is required in determining whether the evidence obtained endorses or refutes the hypothesis.

It appeared that the VLDL were very slightly larger from birds fed the diet containing 55 g/kg maize oil. This increase in size was only marginal and too much importance should not be attached to this single observation, though the finding is supported by the results of Griffin et al. (1982) using diets containing 150 g/kg maize oil. In addition, dietary fat caused the appearance of a considerable number of lipoproteins which were intermediate between the mean portomicron and VLDL size. Portomicrons have been shown to be excluded from the developing oocyte due to their large size (Griffin and Perry, 1985), but it is feasible that these intermediate sized lipoproteins, consisting of small portomicrons and large VLDL, could contribute to yolk formation. Though statistical significance was not attained due to the small number of observations, dietary fat appeared to cause an increase in VLDL triglyceride and this may have been due to these intermediate-sized lipoproteins.

Plasma triglyceride increased in both VLDL and portomicron fractions, despite dietary fat causing functional Lpl activity to increase three-fold, an observation also seen in humans fed a high-fat diet (Frederickson et al., 1963 and Porte et al., 1966). It is assumed that at the time of determining the plasma triglyceride content and PHLA the hen's lipid metabolism was in a steady state, i.e. lipoprotein synthesis = lipoprotein degradation. The increased lipase activity induced by the high-fat diet was insufficient not only to cause a reduction in VLDL size or triglyceride, but also to prevent an accumulation of portomicrons. Hence, the increased portomicron

concentration due to dietary fat could be the cause of the increased VLDL size and abundance and could lead to greater yolk deposition despite increased Lpl activity.

There have been many reports of declining Lpl activity with age in individual tissues (Chlouverakis, 1962 and Zemplenyi, 1962) and PHLA (Bradows and Campbell, 1972). The Lpl activity in adipose tissue of pullets was found to decline from 9 to 20 weeks of age (Pfaff et al., 1977), the authors suggesting the lowered Lpl activity allowed more plasma triglyceride to reach the yolk. Angervall (1960) found that the adipose tissue Lpl activity of 2 year old hens was less than half that of 6 month old birds, though he did not state whether the birds used were broiler or layer strains, nor the sex of the birds. However, in the present experiment, no correlation between PHLA and age was noted in laying hens ranging from 20 to 84 weeks of age. Hence, the evidence is not consistent with the hypothesis that the failure of dietary fat to increase yolk size in older birds is due to a reduction in Lpl activity with age.

In conclusion, there is evidence to support the hypothesis that dietary fat leads to a marginal increase in the concentration and size of plasma VLDL and that this may be associated with increased yolk lipid accumulation, perhaps as a partial consequence of decreased VLDL degradation by Lpl.

SECTION B. THE ROLE OF LIPOPROTEINS IN OVIDUCAL TISSUE METABOLISM

It was shown in Chapter 3 that dietary fat significantly increased albumen weight in both young and old laying hens and that this increase in albumen mass was associated with both higher dry

matter and protein contents. The present section addresses itself to this phenomenon and attempts to identify the biochemical mechanism by which dietary fat induces the oviduct to secrete more albumen protein.

I. INTRODUCTION

On the premise that the first hypothesis to be tested should be the simplest, it was postulated that dietary fat, in the form of lipoproteins, exerts a direct effect on protein synthesis in the oviduct.

The oviduct, and principally the magnum portion, is responsible for the synthesis, storage and secretion of all the 40 or so individual proteins comprising the albumen (O'Malley et al., 1969 and Gilbert, 1976). The possibility that lipid could be involved in some aspect of oviducal function that ultimately enhances protein synthesis is supported by some circumstantial evidence. In terms of wet weight, the oviduct only contains about 2% lipid (Bilgili and Renden, 1985), but the albumen protein synthesising cells of the magnum considerably increase their lipid content as the hen comes into lay (Yu et al., 1972). In addition, Aprahamion et al. (1979) showed that oestrogen administration resulted in a 20-fold increase in oviduct mass and the total oviduct fatty acid synthetase activity increased 180-fold.

One would imagine that the high levels of circulating lipid in the laying hen, particularly VLDL, would be able to provide an adequate supply of lipid to the oviducal tissues. However, because the fatty acid synthetase activity of the oviduct is greatly increased as the hen matures sexually (Aprahamion et al., 1979), it is possible that the oviduct does have a lipid requirement that is not necessarily fulfilled

by the circulatory supply. Perhaps the oviduct has developed a metabolism that is more inclined to use lipid than carbohydrate as a catabolic substrate. Though there is no reported evidence to support this proposition, the role of VLDL in egg formation has already been expounded with reference to yolk deposition, and it is conceivable that perhaps VLDL are important in the metabolism of the oviduct. Dietary fat could increase albumen protein synthesis by supplying additional lipid to the oviducal tissues in the form of VLDL or portomicrons.

Demonstrating that the oviduct possesses the ability to remove a considerable amount of lipid from the circulation would lend credence to the postulation that dietary fat exerts its effect on albumen formation in a direct manner. To this end, the ability of the oviduct to take up lipid was tested indirectly by determining its Lpl activity and comparing it to various other tissues, whilst the uptake of labelled portomicrons, VLDL and unesterified fatty acids (UEFA) gave a direct test.

II. EXPERIMENTAL PROTOCOL

Hens fed a standard layers diet (25 g/kg fat) were used in assessing the oviducal Lpl activity. Acetone-ether extracts were prepared for a variety of tissues and their Lpl activities were determined as described in Chapter 2.

An alternative indication of the importance of lipoproteins in oviducal metabolism was provided by determining the <u>in vivo</u> fate of ^{3}H-portomicrons, ^{14}C-VLDL and ^{14}C-unesterified fatty acid bound to serum albumin (^{14}C-UEFA). Labelled palmitic acid (both ^{3}H- and ^{14}C-) was used in the preparation of the lipoproteins as described in Chapter 2.

The method employed produced nascent newly synthesised labelled VLDL and portomicrons with no cross-labelling (see Fig. 2.1). The label was associated with the triglyceride fraction of the portomicrons (>96%) and the VLDL (>90%).

Fifteen minutes after their injection, the distribution of the labelled lipoproteins between various tissues was determined. The tissues investigated were liver, heart, breast muscle, adipose tissue and the four sections of the oviduct: infundibulum, magnum, isthmus and shell gland. In addition, the three largest unovulated follicles were removed from each bird and the majority of the yolk material was discarded by splitting the follicles while the follicle membranes and newly-deposited yolk material were retained for analysis. Lipid was extracted from the tissues according to the method of Folch et al. (1957) and the radioactivity present determined by liquid scintillation counting employing a double-labelled (^{3}H and ^{14}C) quench curve as described in Chapter 2.

It was anticipated that 15 minutes after injection of the labelled lipoproteins significant quantities of the radioactivity would still be present in the plasma. To distinguish between labelled lipid uptake by tissue cells and the presence of labelled lipid in plasma within the tissue, the plasma volume for each tissue was determined by the method of Bragdon and Gordon (1958) using human ^{125}I-albumin as the blood marker (see Chapter 2).

The fate of the labelled lipoproteins was investigated in two separate trials. One trial involved the simultaneous injection of ^{14}C-UEFA, ^{3}H-portomicrons and ^{125}I-albumin in two hens, whilst the other investigated ^{3}H-portomicrons and ^{14}C-VLDL in four hens.

III. RESULTS

Compared to heart or adipose tissue the four regions of the oviduct did not contain a large amount of Lpl activity (Table 4.3). However, the oviducal tissues had appreciably more Lpl activity than breast muscle, with the magnum exhibiting 10-fold more activity.

In the investigation of the fate of labelled lipoproteins, the blood volume in the tissues of interest were determined in two hens. These values were used in calculating the amount of labelled lipoprotein taken up by each tissue and the results, expressed as a percentage of the label injected into the recipient hens, are given in Table 4.4. Though large amounts of VLDL and UEFA remained in the plasma, after only 15 minutes, over 95% of the injected portomicrons were removed from the circulation. The liver was, by far, the most active tissue in taking up the lipid regardless of the form in which it was presented. Uptake into the heart was also high, conversely, muscle tissue had low uptake of lipid. Adipose tissue exhibited very low uptake of VLDL, but portomicrons and UEFA appeared to be assimilated in appreciable quantities.

The oviducal tissues did not show the ability to remove large quantities of lipid from the circulation in the form of UEFA or portomicrons, though it was higher than the muscle tissue. However, it should be noted that appreciable quantities of lipid in the form of VLDL were taken up by the oviducal tissues. This preference for VLDL is reflected in the high ratio of VLDL:portomicron exhibited by the oviducal tissues which was higher than for other tissues studied, except for follicle membranes (Table 4.4). As expected, large amounts of VLDL were deposited in the follicles, but it was interesting to note that portomicrons were also taken up.

Table 4.3: The lipoprotein lipase activities (+ SEM) of various tissues, including the oviducal regions

Tissue	N	Lipoprotein Lipase Activity (μmol/hr/g tissue)
Heart	8	50.6±1.8
Adipose	4	15.4±3.0
Breast Muscle	4	0.3±0.3
Infundibulum	4	5.4±3.3
Magnum	8	2.5±1.0
Isthmus	4	2.1±1.3
Shell Gland	4	5.9±2.7

EXPERIMENTAL DETAILS

Tissues were obtained from IsaBrown hens fed a standard layers diet (25g fat/kg). Acetone-ether extracts were prepared for the tissues and the Lpl activities determined on these extracts as described in Chapter 2.

Table 4.4: The plasma volumes of various tissues and the distribution of labelled lipoproteins between the tissues 15 minutes after their administration, expressed as a percentage (+ SD) of the total label injected per bird

Tissue	Plasma Volume (μl/g)	^{14}C-UEFA (%/g)	^{3}H-portomicrons (%/g)	^{14}C-VLDL (%/g)	Ratio (VLDL: portomicron
(Observations)	(2)	(2)	(6)	(4)	
Heart	65.6± 6.3	0.344± .035	0.056± .020	0.115± .054	2.05
Liver	110.3± 0.6	0.901± .309	1.332± .332	1.050± .087	0.79
Breast Muscle	4.8± 2.2	0.010± .001	0.003± .001	0.004± .002	1.32
Adipose	7.2± 0.2	0.014± .101	0.014± .009	0.006± .017	0.44
Follicle	11.0± 4.0	0.065± .016	0.091± .014	0.647± .151	7.09
Infundibulum	43.9±16.2	0.034± .008	0.013± .002	0.044± .008	4.90
Magnum	26.0± 6.6	0.026± .005	0.009± .002	0.034± .008	3.76
Isthmus	24.7± 2.0	0.018± .003	0.007± .001	0.018± .025	2.50
Shell Gland	25.1± 0.5	0.020± .003	0.020± .009	0.084± .049	4.12
Plasma (/ml)		0.232± .070	0.024± .008	0.281± .087	11.70
Total Plasma		38.83±8.72	4.072±1.59	47.04±12.46	11.55

EXPERIMENTAL DETAILS

Birds used were IsaBrown hens, aged 36 weeks, reared on a standard layer's diet (25g fat/kg). Labelled palmitic acid was used in the preparation of ^{3}H-portomicrons, ^{14}C-VLDL and ^{14}C-unesterified fatty acid bound to serum albumin (^{14}C-UEFA). Fifteen minutes after their injection, the distribution of the labelled lipoproteins between various tissues was determined by liquid scintillation counting following lipid extraction. The plasma volume for each tissue was determined using ^{125}I-albumin as the blood marker to distinguish between labelled lipid uptake and the presence of labelled lipid in plasma within the tissue. Full experimental datails are given in Chapter 2.

The fate of the labelled lipoproteins was investigated in two separate trials. One trial involved the simultaneous injection of ^{14}C-UEFA, ^{3}H-portomicrons and ^{125}I-albumin in two hens, whilst the other investigated ^{3}H-portomicrons in four hens.

IV. DISCUSSION

An indication of the importance that lipid plays in oviducal function was assessed by determining both the Lpl activity and the uptake of labelled lipoproteins in the oviduct relative to other tissues. Though the oviduct contained less Lpl activity (units/g tissue) than heart or adipose tissue, the activity (units/g tissue) was considerably higher than that of breast muscle tissue. This would suggest that the oviduct has the ability to supply itself with large amounts of lipid by the action of Lpl on triglyceride-rich lipoproteins. A similar view is suggested by the relatively high uptake of labelled lipoproteins, especially VLDL, by oviducal tissues.

It was of interest to investigate UEFA as a lipid source in the hen because the differences between avian and mammalian lipid metabolism could mean that UEFA bound to serum albumin is an important mode of lipid transport in fat-fed birds. The absence of a developed lymphatic system in the hen means that dietary fat enters directly into the portal blood rather than via the lymph. It is well known that some dietary fat, especially short chain fatty acids, does not pass into the lymph but enters the portal blood directly bound to serum albumin (Freeman, 1976). Obviously, with no lymphatic system this route of lipid transport could be very important to avians. It was found that 39% of labelled UEFA was still present in the blood after 15 minutes, though Bragdon & Gordon (1958) reported that after 10 minutes only 1% of labelled UEFA remained in the circulation of rats. It is possible that in the present investigation, a large amount of the radioactivity present in the hen plasma was not UEFA but in fact was VLDL that had been synthesised by the liver after removing UEFA from the circulation.

The high percentage of labelled VLDL still present in the circulation after 15 minutes supports the belief that mature VLDL are resistant to degradation and are designed primarily for yolk deposition. Bacon et al. (1978) showed that 75% of labelled hen VLDL remained in the circulation after 100 minutes, whilst only 6% of labelled immature VLDL was not removed. In contrast to VLDL's low susceptibility, only 4% of the labelled portomicrons in the present experiment remained in the circulation after 15 minutes. The much shorter half-life of the portomicron is presumably due to its high Lpl-activiting apoprotein content. This in vivo demonstration of Lpl's preferential action on portomicrons lends support to the hypothesis that an increase in portomicron concentration could result in a decreased degradation of VLDL (see Chapter 4 Section A).

It is not surprising to find that large amounts of VLDL lipid had been deposited in the follicle, and the high ratio of VLDL: portomicron supports the idea that mature hen VLDL are synthesised for the purpose of yolk deposition. Despite seven times more ^{14}C-VLDL lipid being deposited in the follicle, a considerable amount of ^{3}H-portomicron lipid was also detected. This observation was unexpected as portomicrons are unable to enter the oocyte due to their large size (Griffin and Perry, 1985, also see electron micrographs Chapter 6). Bensadoun and Kompiang (1979) showed that labelled portomicron lipid was incorporated into the follicle one hour after administration, but it is strongly suspected that the label detected in the follicles had been deposited in the form of resynthesised VLDL lipid. In the present experiment, the relatively large amount of labelled portomicron lipid found in the follicle membrane tissues could

also have been in the form of VLDL, though 15 minutes for the hepatic uptake of labelled portomicrons, synthesis and secretion of labelled VLDL does appear rather rapid. The possible role that portomicrons trapped in the follicle membrane may play in steroidogenesis is discussed in Chapter 6.

As was mentioned above, all four regions of the oviduct assimilated relatively large quantities of lipid in any form it was presented compared to breast muscle tissue, but much less than heart and liver tissue. It is interesting to note that the ratio of VLDL:portomicron lipid taken up by the oviduct was high. Liver, heart and muscle tissue appeared to utilise both VLDL and portomicrons equally, whilst adipose tissue showed a slight preference for portomicrons, but oviducal tissue seems to be adapted to take up four-fold more VLDL lipid than that from portomicrons. Why a tissue should preferentially utilise lipid from a less accessible source in the form of the resistant VLDL rather than the more susceptible portomicrons is difficult to comprehend. This phenomenon is very curious and merits further study. It is suggested that the oviduct may have developed a method of receptor-mediated endocytosis for the uptake of mature hen VLDL.

The role of lipid in the formation of albumen proteins is not obvious. The finding that oestrogen treatment greatly increases the fatty acid synthetase activity in the hen oviduct led Aprahamion et al. (1979) to suggest that fatty acid synthetase makes an important contribution to the large lipid requirement of the developing egg though an explanation for the role of lipid in the oviduct was not offered. The molecular biology of protein synthesis in eukaryotic

cells is well established and documented (for a recent review see Moldave, 1985) and it would appear that lipid plays a negligible role. However, there is some published evidence that in the formation of albumen proteins, lipid is involved in the initial stages of protein synthesis.

In a series of papers, Hendler (1959, 1962a, 1963a, 1963b and 1965) demonstrated in the hen oviduct the production of lipid-amino acid complexes, apparently linked by covalent bonds. The addition of chemical agents known to be detrimental to the formation of these lipid-amino acid complexes resulted in the potent inhibition of amino acid incorportion into protein. The extensive work of Hendler in this area led him to propose an alternative model for protein synthesis applicable, at least, to the hen oviduct (Hendler, 1962b), in which lipid plays an important role.

Nearly all of the albumen proteins are glycosylated to some degree resulting in carbohydrate forming over 5% (w/w) of the albumen protein (calculated from data of Baker, 1968 and Feeney and Allison, 1969). Cumulative evidence from the studies of several laboratories has implicated that an oligosaccharide-lipid intermediate is involved in the post-translational glycosylation process in several *in vitro* systems such as pig liver microsomes (Brehens *et al.*, 1973); mouse myeloma cells (Hsu *et al.*, 1974) and human lymphocyte membranes (Wedgwood *et al.*, 1974). Lipid has also been demonstrated to act as an intermediate in the glycoprotein synthesis in the hen oviduct (Waechter *et al.*, 1973; Lucas *et al.*, 1975 and Struck and Lennarz, 1976).

As can be seen from the above, lipid has been implicated in the formation of the albumen proteins from the initial stages of protein biosynthesis to the post-translational modification. The role that dietary fat may play in increasing the formation and secretion of albumen proteins, however, is not so clear. One would imagine that the high levels of circulating lipid in the laying hen, particularly VLDL, would be able to provide adequate supplies of the lipid involved in albumen protein formation by the processes described above, especially as the lipid is recycled presumably. Alternatively, it is possible that the oviduct has developed a metabolism that preferentially uses lipid as a catabolic substrate.

In summary, it can be concluded that there does exist a plausible role for lipid in albumen protein synthesis. The oviduct appears to have a relatively large requirement for lipid, which is reflected in its Lpl activity and the uptake of lipid in the form of the lipoproteins UEFA and portomicrons, but namely VLDL. It was noted that the oviduct utilises more VLDL lipid than portomicron lipid, and it is suggested that this constant lipid source, peculiar to egg formation, could supply the oviducal tissue with its relatively large lipid requirement. Fat-feeding has been shown to increase VLDL size and concentration (see Chapter 4 Section A) and thus supplying more lipid for oviducal uptake. In this manner, dietary fat may exert its effect on egg size via increased albumen formation.

SECTION C. THE EFFECT OF DIETARY FAT UPON PLASMA OESTROGEN LEVELS

The previous two sections attempted to explain the effect of dietary fat upon egg weight by investigating mechanisms involving either yolk or albumen production separately. In contrast, attention will now be paid to a possible mechanism which could influence both yolk and albumen weight - increased levels of circulating oestrogen.

I. INTRODUCTION

Both yolk and albumen formation were briefly reviewed in Chapter 1 and it was noted that steroid hormones, especially oestrogen, play an important role. Under the influence of oestrogen, VLDL is affected both quantitatively (Yu et al., 1976) and qualitatively (Kudzama et al., 1979). Kudzama et al., (1975) demonstrated that hepatic triglyceride synthesis was greatly stimulated in immature chicks treated with diethylstilbestrol, while increased phospholipid biosynthesis in similarly treated roosters liver was also reported (Vigo and Vance, 1981). Simultaneously with lipid synthesis, the apoprotein components of VLDL were also stimulated by oestrogen treatment (Wiskocil et al., 1980) to produce lipoproteins high in apo-B and apo-VLDL-II. The synthesis of many other yolk proteins not associated with VLDL are also influenced by oestrogen, namely vitellogenin for which the corresponding mRNA increases several thousand-fold in diethylstilbestrol treated roosters (Deeley et al., 1977).

The oviduct of immature pullets is greatly increased in weight when treated singly with oestrogen, progesterone or testosterone, but oestrogen in combination with either progesterone or testosterone is required for inducing morphological and physiological development of

the magnum (Brant and Nalbandov, 1956). After oestrogen stimulation the mucosa of the magnum differentiates into three distinct cell-types: a ciliated cell-type concerned with motility: and two albumen protein synthesising cell-types, the tubular gland cell and the goblet cell. Under the influence of oestrogen, the tubular gland cell produces most of the 40 or so albumen proteins including the cell specific proteins ovalbumin and lysozyme, whilst the goblet cell is stimulated by progesterone to produce just one protein, avidin. Continued administration of oestrogen is required to maintain cell differentiation and albumen protein synthesis (Schimke et al., 1973).

As can be seen from the above, the steroid hormones, but especially oestrogen, are crucial to the production of yolk precursors and albumen proteins. Both yolk and albumen weight have been demonstrated to increase in response to dietary fat (Chapter 3), Experimental evidence exists to indicate that dietary fat might affect circulating levels of steroid hormones. Talavera et al. (1985) reported that dietary fat significantly increased plasma progesterone levels in the mid- to late-luteal phase of the oestrus cycle in Holstein heifers, though oestrogen levels were not affected.

Although dietary fat has been shown to affect progesterone but not oestrogen concentrations (Talavera et al., 1985), it was decided to investigate the effect of fat-feeding on oestrogen levels in hens. Progesterone was not chosen because even if dietary fat did affect its circulating levels it would not easily explain how yolk nor albumen weight was increased since progesterone is not reported to play a major part in yolk formation and it is only responsible for the synthesis of one albumen protein, avidin, which comprises less than 0.05% (w/w) of

albumen (Gilbert, 1976). On the other hand, it has already been explained the major role that oestrogen plays in both yolk and albumen formation. Thus, the effect of dietary fat upon oestrogen levels was investigated.

II. EXPERIMENTAL PROTOCOL

The birds used to investigate the effect of dietary fat on plasma oestrogen levels were those from the second nutritional trial (Chapter 3, EXPERIMENT 2), aged 42 weeks old. Only birds that were noted to have laid an egg on the day of blood sampling and on the preceding and following day were included in the oestrogen determination. Oestradiol-17β was measured by a double antibody radioimmunoassay following an initial purification (see Chapter 2).

The assay's precision, accuracy, sensitivity and the solvent interference were determined in order to validate the method.

III. RESULTS

The assay's reproducibility, measured by calculating the within-assay coefficient of variation on 10 pooled plasma samples, was 8.2%. Accuracy was determined by comparing the amount of hormone measured for various amounts of cold oestradiol with the amount actually added to pooled plasma samples, a correlation coefficient of 0.995 was obtained. Assay sensitivity, defined as the concentration of hormone needed to give a response two standard deviation units higher than the zero standard response, was 0.7 pg. The average solvent blank value was 8.1 pg, and this value was subtracted from all the determined plasma values.

Table 4.5: The effect of dietary fat on the plasma concentrations of oestradiol-17β (Mean + SEM)

Dietary Treatment	N	Oestradiol concn (pg/ml)	P[1]
Low-fat	14	238.2±16.8	<0.01
High-fat	15	307.9±18.3	

[1] Means separated by Student's t-test

EXPERIMENTAL DETAILS

Birds were fed either the the low- or the high-fat diet (see Table 3.1 for composition) from week 23 until the blood sampling, week 42. The blood sampling was performed between 08.00-10.00 hrs on one day. Only birds that were noted to have laid an egg on the day of blood sampling and on the preceding and following day were included in the oestrogen determination. Oestradiol-17β was measured by a double antibody radioimmunoassay following an initial purification (see Chapter 2).

The effect of dietary fat on circulating oestradiol levels is shown in Table 4.5. Plasma from birds fed the high-fat diet contained significantly ($P<0.01$) more oestradiol, by almost a third, than those fed the low-fat diet.

IV. DISCUSSION

With the increased concern about the carcinogenic property of benzene, a method was developed to purify oestradiol from lipid-rich hen plasma with a toluene-based solvent system and gel filtration on Sephadex-LH20 mini-columns. The assay's validation was well within or bettered the range reported by other workers (Peterson and Common, 1972; Johnson and van Tienhoven, 1980; Hagan et al., 1984 and Leszczynski et al., 1984) and ensured the reliability of the present findings.

The high-fat diet, 55 g/kg maize oil, caused a significant increase in circulating oestradiol levels when compared to an isoenergetic and isonitrogenous low-fat diet. The possible mechanisms by which this phenomenon occurs are fully discussed in Chapter 6. The higher oestrogen levels may increase egg weight in fat-fed birds by stimulating the hepatic synthesis of proteins and lipids involved in yolk formation and by increasing albumen protein production in the oviduct.

CHAPTER 5

THE EFFECT OF VARIOUS FAT TYPES AND INCLUSION LEVELS ON LAYING HEN PERFORMANCE

I. INTRODUCTION

Previous studies on the effects of different types of dietary fat on egg weight were discussed in Chapter 1 and it was noted that the reported responses were extremely variable (Table 1.2). It is difficult to reconcile such conflicting results. Unfortunately, there are two aspects of these earlier experiments which impede the interpretation and comparisons of the results. Firstly, there have been few reports of two or more dietary fats being evaluated in the same experiment. Hence, the only comparisons of egg weight response to different fat types that are available are those between the results of different experiments which must be compromised by dissimilar experimental conditions such as: diet composition, age (see Chapter 3) and strain of birds; and the rearing and husbandry of the birds. Secondly, only rarely did earlier workers investigate the effect of dietary fat at several inclusion levels on egg weight. It is apparent that more detailed information is required on the response of egg weight to a variety of dietary fats each fed at several inclusion levels.

A large-scale experiment was designed to investigate the response of egg weight to a variety of dietary fat types differing in fatty acid chain length and degree of saturation fed at several inclusion levels. The choice of fats also allowed the effect of linoleic acid per se on egg weightto be determined. In addition, the experiment investigated whether dietary fats other than maize oil increased egg weight via both yolk and albumen weight in young birds (see Chapter 3) and whether these changes were accompanied by changes in plasma oestrogen levels (see Chapter 4).

II. EXPERIMENTAL DESIGN

A. Experimental Protocol

One thousand two hundred and forty eight Isa Brown female chicks were reared according to the standard pullet replacement procedures described in Chapter 2, except for the lighting regime. Up to the age of 18 weeks the birds were subjected to 8 hours of light a day; thereafter, daylength was gradually increased to a maximum of 17 hours over the next twelve weeks. All of the birds were fed a low-fat diet from 17 to 24 weeks of age. Egg weights and food consumption for each bird were determined during the 24th week.

The birds, grouped in 104 experimental units of 12 hens each, were assigned to the 19 dietary treatments using random number tables. Five experimental units were allocated to each of the 18 diets of different fat types and inclusion levels, whilst the remaining 14 experimental units were maintained on the low-fat diet. The extra replication on the low-fat diet ensured an accurate determination of the birds' response to a low-fat diet which would be used for the zero fat inclusion level (0 g/kg) for each of the five fat types. The birds were fed their allocated dietary treatment for 5 weeks up to the age of 29 weeks. The egg weights and food consumption for each bird were determined during the 29th week.

Eggs were collected on two consecutive days during week 29 from half of the birds on the low-fat diet, all of the birds on the maximum inclusion level diets containing tallow, fish oil and coconut oil, and all of the birds fed the maize oil diets. The eggs were stored at 4°C and the yolk, albumen and shell weights from one egg per bird were determined within 10 days of collection.

On the last day of the 29th week, blood samples were taken from 28 birds on each of ten of the dietary treatments (see Table 5.14) for oestradiol determinations. The birds chosen were known to have been laying regularly and only samples from those birds which had laid an egg on that day and on the preceeding and following days were included in the plasma oestradiol determination. Oestradiol was measured by RIA as described in Chapter 2.

B. Diets

The dietary fats used were chosen to test the response of egg weights to relatively commonly used fats containing fatty acids differing in both chain lengths and the degree of saturation (see Table 5.1). Coconut oil was chosen as a source of short chain saturated fatty acids, mainly lauric acid. Tallow is a source of medium chain saturated fatty acids, principally palmitic and stearic acid. Maize oil had been the fat of choice to investigate the effect of dietary fat on egg size previously (see Chapters 3 and 4) and was used in the present experiment as a source of medium chain unsaturated fatty acids, mainly linoleic acid. A mixture of menhaden and horse mackerel fish oil at a ratio of 4:3 (w/w) was used (as a source of long chain polyunsaturated fatty acid).

The controversy as to whether linoleic acid has a greater influence upon egg size than other readily absorbable dietary fats was discussed in Chapter 1. Diets containing olive oil, high in oleic acid, were included in the experiment to investigate this issue.

Table 5.1: Fatty acid profile (%) of fats used in experimental diets

Fatty Acid Name	Shorthand Notation	Position of terminal double bond	Coconut Oil	Tallow	Olive Oil	Maize	Menaden: Horse Mackerel Oils (4:3)
Capric	10:0		2				
Lauric	12:0		59				
Myristic	14:0		18				7
Palmitic	16:0		10	34	6	12	24
Stearic	18:0		2	37	4	3	6
Palmitoleic	9-16:1			3		1	8
Oleic	9-18:1		8	25	83	27	20
Gondoic	11-20:1		1				1
Erucic	13-22:1						
α-Linoleic	9,12-18:2	n-6	1	1	7	56	3
Arachidonic	5,8,11,14-20:4	n-6					2
α-Linolenic	9,12,15-18:3	n-3				1	3
Octadecatetraenoic	6,9,12,15-18:4	n-3					3
Eicosapentaenoic	5,8,11,14,17-20:5	n-3					2
Docosapentaenoic	7,10,13,16,19-22:5	n-3					2
Docosahexaenoic	4,7,10,13,16,19-22:6	n-3					7

The inclusion levels chosen for the experiment were approximately 0, 10, 20, 40 and 60 g/kg. It was important to ensure that possible differences in egg weight response due to the dietary treatments could not be assigned to slight variations in the metabolisable energy (ME) content of the treatments arising from the dissimilar ME contents of the fats themselves and their differing degrees of absorbability. Maize oil was assigned an ME value of 36.61 kJ/g (calculated from Renner and Hill, 1960; Young, 1961; and Reid, 1984) and the amounts of the other fats were calculated to provide the same ME at the broadly equivalent inclusion levels. The fish oil was assigned an ME value of 35.93 kJ/g (calculated from Opstvedt, 1985) and with a high absorbability, the ME value was assumed to be unchanged at all inclusion levels. The wide range of reported energy contents (29-38 kJ/g) of animal fat is presumably due to inter-batch variation. It was decided to assume an absorbability of 92% for tallow at the 10 g/kg inclusion level (Renner and Hill, 1960 and 1961 and Young, 1961) and its gross energy was determined to be 39.41 kJ/g using bomb calorimetry (kindly performed by K. Henderson, IGAP). Saturated fats are known to be less readily absorbed at high inclusion levels (Freeman, 1976) and Wiseman and Lessire (1988) have quantified the subsequent reduction in the ME values. Hence, though the tallow was assigned an ME value of 36.26 kJ/g at the 10 g/kg inclusion level, an ME value of 35.00 kJ/g was assigned at the 60 g/kg inclusion level. Coconut oil was assumed to have an ME value of 35.98 kJ/g (Scott et al., 1982) at the 10 g/kg inclusion level, but this was assumed to fall to 35.50 kJ/g at the 60 g/kg inclusion level.

Olive oil was assigned the same ME value as maize oil and it was assumed that this value would be the same at the two inclusion levels tested, 20 and 60 g/kg.

The diets were offered ad libitum in the form of mash and were formulated to be both isonitrogenous and isoenergetic. Their compositions are given in Tables 5.2-5.6

C. Data Analysis

The effects of dietary treatment on various aspects of laying performance were examined by one-way analysis of variance. If a significant effect was demonstrated, then two-way analysis of variance was employed to partition the response between the fat type and inclusion level. Data for the low-fat and olive oil diets were excluded from the two-way analysis of variance in order to create a balanced 4 x 4 factorial experimental design, consisting of four fat types and four inclusion levels.

According to Mead and Pike (1975) the experiment was most suited to model fitting by response surface methodology. For each fat type, excluding olive oil, polynomial response functions were fitted and tested by the reduction in the residual sum of squares. The regression coefficients for the different fats were compared using least significant difference following an estimation of their variances which allowed for the covariant component (Steel and Torrie, 1980).

Unlike the experiments described in Chapter 3, the results for the egg components measured in the present experiment were obtained post-treatment only and the change in the egg component due to treatment was not investigated. The significance of the effect of dietary

Table 5.2: Composition of experimental diets containing maize oil

	Fat inclusion level (g/kg diet)				
	0	10	20	40	60
Ingredient (g/kg)					
Wheat	756.0	681.2	649.0	468.0	344.0
Soya meal	32.5	60.5	72.0	85.0	94.0
Herring meal	67.5	72.5	65.6	60.0	55.0
Meat & bone meal	23.0	21.0	21.0	20.0	20.0
Oats	-	-	9.5	71.2	117.0
Barley	-	25.0	9.0	60.0	75.0
Wheatfeed	-	12.5	16.5	33.0	36.0
Dried grass meal	10.0	20.0	40.0	64.0	100.0
Limestone flour	70.0	71.5	69.7	71.0	71.2
Dicalcium phosphate	18.2	18.0	20.0	20.0	20.0
Protein supplement[a]	15.0	-	-	-	-
Sodium chloride	2.8	2.8	2.8	2.8	2.8
Vitamin supplement[b]	2.5	2.5	2.5	2.5	2.5
Mineral supplement[b]	2.5	2.5	2.5	2.5	2.5
Maize oil	-	10.0	20.0	40.0	60.0
Calculated composition (per kg)					
Metabolisable energy (MJ)	11.34	11.34	11.34	11.34	11.34
Protein (g)	169(163)[c]	169(162)	169(161)	169(163)	169(161)
Available phosphorus (g)	6.3(8.0)[d]	6.3(8.5)	6.6(9.3)	6.4(8.4)	6.3(9.1)
Ether extractives (g)	22(18)	30(28)	40(43)	60(66)	80(92)
Linoleic acid (g)	6.5	12.1	17.5	28.8	39.2

[a] FPD-950 (95% protein), I.C.I. England
[b] Vitamin and mineral supplement: composition given in Table 3.1
[c] Figures in parentheses are determined analyses
[d] Determined analyses are total phosphorus.

Table 5.3: Composition of experimental diets containing fish oil

	Fat inclusion level (g/kg diet)				
	0	10	20	40	60
Ingredient (g/kg)					
Wheat	756.0	681.2	649.0	468.0	344.0
Soya meal	32.5	60.5	72.0	85.0	94.0
Herring meal	67.5	72.5	65.6	60.0	55.0
Meat & bone meal	23.0	21.0	21.0	20.0	20.0
Oats	-	-	9.5	71.0	117.0
Barley	-	25.0	9.0	60.0	75.0
Wheatfeed	-	12.5	16.5	33.0	36.0
Dried grass meal	10.0	20.0	40.0	64.0	100.0
Limestone flour	70.0	71.5	69.5	71.5	70.5
Dicalcium phosphate	18.2	18.0	20.0	20.0	19.5
Protein supplement[a]	15.0	-	-	-	-
Sodium chloride	2.8	2.55	2.5	2.5	2.5
Vitamin supplement[b]	2.5	2.5	2.5	2.5	2.5
Mineral supplement[b]	2.5	2.5	2.5	2.5	2.5
Fish oil	-	10.25	20.5	41.0	61.5
Calculated composition (per kg)					
Metabolisable energy (MJ)	11.34	11.34	11.34	11.34	11.34
Protein (g)	169(163)[c]	169(161)	169(160)	169(162)	169(160)
Available phosphorus (g)	6.3(8.0)[d]	6.3(7.7)	6.5(8.1)	6.4(8.7)	6.2(7.7)
Ether extractives (g)	22(18)	30(32)	40(39)	60(66)	80(92)
Linoleic acid (g)	6.5	6.8	6.9	7.6	8.0

[a] FPD-950 (95% protein) I.C.I. England
[b] Vitamin and mineral supplement composition given in Table 3.1
[c] Figures in parentheses are determined analyses
[d] Determined analyses are total phosphorus.

Table 5.4: Composition of experimental diets containing tallow

	Fat inclusion level (g/kg diet)				
	0	10	20	40	60
Ingredient (g/kg)					
Wheat	756.0	681.2	649.0	468.0	344.0
Soya meal	32.5	60.5	72.0	85.0	94.0
Herring meal	67.5	72.5	65.6	60.0	55.0
Meat & bone meal	23.0	21.0	21.0	20.0	20.0
Oats	-	-	9.5	71.2	117.0
Barley	-	25.0	9.0	60.0	75.2
Wheatfeed	-	12.5	16.5	33.0	36.0
Dried grass meal	10.0	20.0	40.0	64.0	100.0
Limestone flour	70.0	71.4	69.1	69.8	70.2
Dicalcium phosphate	18.2	18.0	20.0	20.0	18.5
Protein supplement[a]	15.0	-	-	-	-
Sodium chloride	2.8	2.7	2.5	2.8	2.8
Vitamin supplement[b]	2.5	2.5	2.5	2.5	2.5
Mineral supplement[b]	2.5	2.5	2.5	2.5	2.5
Tallow	-	10.2	20.9	41.2	62.4
Calculated composition (per kg)					
Metabolisable energy (MJ)	11.34	11.34	11.34	11.34	11.34
Protein (g)	169(163)[c]	169(162)	169(162)	169(162)	169(162)
Available phosphorus (g)	6.3(8.0)[d]	6.5(9.6)	6.5(8.3)	6.4(8.3)	6.1(8.4)
Ether extractives (g)	22(18)	40(40)	40(40)	60(61)	80(88)
Linoleic acid (g)	6.5	8.1	8.1	10.0	11.6

[a] FPD-950 (95% protein) I.C.I. England
[b] Vitamin and mineral supplement composition given in Table 3.1
[c] Figures in parentheses are determined analyses
[d] Determined analyses are total phosphorus.

Table 5.5: Composition of experimental diets containing coconut oil

	Fat inclusion level (g/kg diet)				
	0	10	20	40	60
Ingredient (g/kg)					
Wheat	756.0	681.2	649.0	468.0	344.0
Soya meal	32.5	60.5	72.0	85.0	94.0
Herring meal	67.5	72.5	65.5	60.0	55.0
Meat & bone meal	23.0	21.0	21.0	20.0	20.0
Oats	-	-	9.5	71.2	117.0
Barley	-	25.0	9.0	60.0	75.2
Wheatfeed	-	12.5	16.5	33.0	36.0
Dried grass meal	10.0	20.0	40.0	64.0	100.0
Limestone flour	70.0	71.3	69.5	70.0	69.0
Dicalcium phosphate	18.2	18.0	20.0	20.0	20.0
Protein supplement[a]	15.0	-	-	-	-
Sodium chloride	2.8	2.8	2.5	2.8	2.8
Vitamin supplement[b]	2.5	2.5	2.5	2.5	2.5
Mineral supplement[b]	2.5	2.5	2.5	2.5	2.5
Coconut Oil	-	10.2	20.5	40.9	62.0
Calculated composition (per kg)					
Metabolisable energy (MJ)	11.34	11.34	11.34	11.34	11.34
Protein (g)	169(163)[c]	169(161)	169(163)	169(164)	169(165)
Available phosphorus (g)	6.3(8.0)[d]	6.3(8.0)	6.5(8.4)	6.4(8.2)	6.3(7.7)
Ether extractives (g)	22(18)	30(30)	40(43)	60(61)	80(87)
Linoleic acid (g)	6.5	6.8	6.8	7.4	7.7

[a] FPD-950 (95% protein) I.C.I. England
[b] Vitamin and mineral supplement composition given in Table 3.1
[c] Figures in parentheses are determined analyses
[d] Determined analyses are total phosphorus.

Table 5.6: Composition of experimental diets containing olive oil

	Fat inclusion level (g/kg diet)		
	0	20	60
Ingredient (g/kg)			
Wheat	756.0	649.0	344.0
Soya meal	32.5	72.0	94.0
Herring meal	67.5	65.6	55.0
Meat & bone meal	23.0	21.0	20.0
Oats	-	9.5	117.0
Barley	-	9.0	75.0
Wheatfeed	-	16.5	36.0
Dried grass meal	10.0	40.0	100.0
Limestone flour	70.0	69.7	71.2
Dicalcium phosphate	18.2	20.0	20.0
Protein supplement[a]	15.0	-	-
Sodium chloride	2.8	2.8	2.8
Vitamin supplement[b]	2.5	2.5	2.5
Mineral supplement[b]	2.5	2.5	2.5
Olive oil	-	20.0	60.0
Calculated composition (per kg)			
Metabolisable energy (MJ)	11.34	11.34	11.34
Protein (g)	169(163)[c]	169(160)	169(161)
Available phosphorus (g)	6.3(8.0)[d]	6.5(8.1)	6.3(9.0)
Ether extractives (g)	22(18)	40(40)	80(77)
Linoleic acid (g)	6.5	7.7	10.4

[a] FPD-950 (95% protein) I.C.I., England
[b] Vitamin and mineral supplement composition given in Table 3.1
[c] Figures in parentheses are determined analyses
[d] Determined analyses are total phosphorus

treatment on the various egg components and plasma oestrogen was tested by one-way analysis of variance and Duncan's multiple range test (Duncan, 1955). The relationships between increasing inclusion levels of dietary maize oil and the egg component weights were investigated by calculating correlation coefficients.

III. RESULTS

Over the 5 week experimental period there was a slight increase in daily food intake (Table 5.7) though this was not significantly ($P>0.1$) affected by the dietary treatment. However, the 60 g/kg inclusion level for each of the fat types tended to cause a slight reduction in food intake.

Egg weight increased by 3.5 g in the control group during the 5 week experiment (Table 5.8), and preliminary one-way analysis of variance showed that dietary treatment significantly ($P<0.05$) affected this increase. Two-way analysis of variance revealed that the effect was due to both the fat type ($P<0.001$) and the inclusion level ($P<0.005$) and the presence of a significant interaction ($P<0.005$) between these two factors would indicate that they do not exert their effect upon egg weight independently.

Response functions for the effect of the different fats on egg weight were fitted (Fig. 5.1) and were all of quadratic form. The linear and quadratic regression coefficients for the fat types were compared (Table 5.9). No two fats had the same quadratic function ($P<0.05$). Hence, the egg weight response appeared to be a unique characteristic of the fat type. The optimal inclusion level for all of the fat types investigated was below 60 g/kg, with egg weights depressed when fish oil was fed in excess of 20 g/kg.

Table 5.7: The effect of dietary treatment upon the change in the daily food intake (g + SD) over the 5 week experimental period (food consumed during week 29 - food consumed during week 24)

Fat Inclusion Level / Fat Type	0 g/kg (n=14)[1]	10 g/kg (n=5)	20 g/kg (n=5)	40 g/kg (n=5)	60 g/kg (n=5)
Maize Oil	5.668 ±6.496	3.801 ±3.230	7.413 ±5.890	7.442 ±7.480	1.555 ±3.867
Fish Oil	5.668 ±6.496	8.018 ±1.343	1.637 ±2.846	8.915 ±4.570	-1.699 ±3.857
Tallow	5.668 ±6.496	4.786 ±2.606	1.701 ±2.721	5.846 ±3.079	2.286 ±5.027
Coconut Oil	5.668 ±6.496	6.425 ±1.408	2.823 ±4.540	1.437 ±2.887	0.553 ±4.450

	Significance of treatment effect	LSD (P=0.05)
Dietary treatment	>0.1	7.62

[1] Experimental group is 12 birds, i.e. N=14 is 14 groups of 12 birds

EXPERIMENTAL DETAILS

All birds were fed the low-fat diet (0g fat/kg) from 17 to 24 weeks of age. The birds were then allocated to their appropriate dietary treatment from week 25 to 29. Food consumption was determined during the 24th week and again during the 29th week.

Table 5.8: The effect of dietary treatment upon the change in egg weight (g + SD) over the 5 week experimental period (egg weight week 29 - egg weight week 24)

Fat Type \ Fat Inclusion Level	0 g/kg (n=14)[1]	10 g/kg (n=5)	20 g/kg (n=5)	40 g/kg (n=5)	60 g/kg (n=5)
Maize Oil	3.468 ± .628	4.787 ± .643	5.114 ± .361	6.201 ± .680	6.361 ± .446
Fish Oil	3.468 ± .628	3.541 ± .633	4.003 ± .573	3.664 ± .428	2.058 ± .464
Tallow	3.468 ± .628	4.533 ± .784	4.709 ± .815	4.992 ± .629	4.819 ± .834
Coconut Oil	3.468 ± .628	3.793 ± .555	3.754 ±1.088	4.969 ± .572	4.349 ± .528
Olive Oil	3.468 ± .628		4.625 ± .709		4.468 ± .509

	Significance of treatment effect	LSD (P=0.05)
Dietary treatment	<0.005	0.803
Fat type	<0.001	0.813
Fat level	<0.005	0.813
Type x level	<0.005	1.626

[1] Experimental group is 12 birds, i.e. N=14 is 14 groups of 12 birds

EXPERIMENTAL DETAILS

All birds were fed the low-fat diet (0g fat/kg) from 17 to 24 weeks of age. The birds were then allocated to their appropriate dietary treatment from week 25 to 29. Egg weights were determined during the 24th week and again during the 29th week.

Fig 5.1:The fitted quadratic curves that describe the increase in egg weight (±SEM) over a 5-week period in hens fed various levels of maize oil(●—●),tallow(△--△),coconut oil(○-·-○),or fish oil (▲···▲).

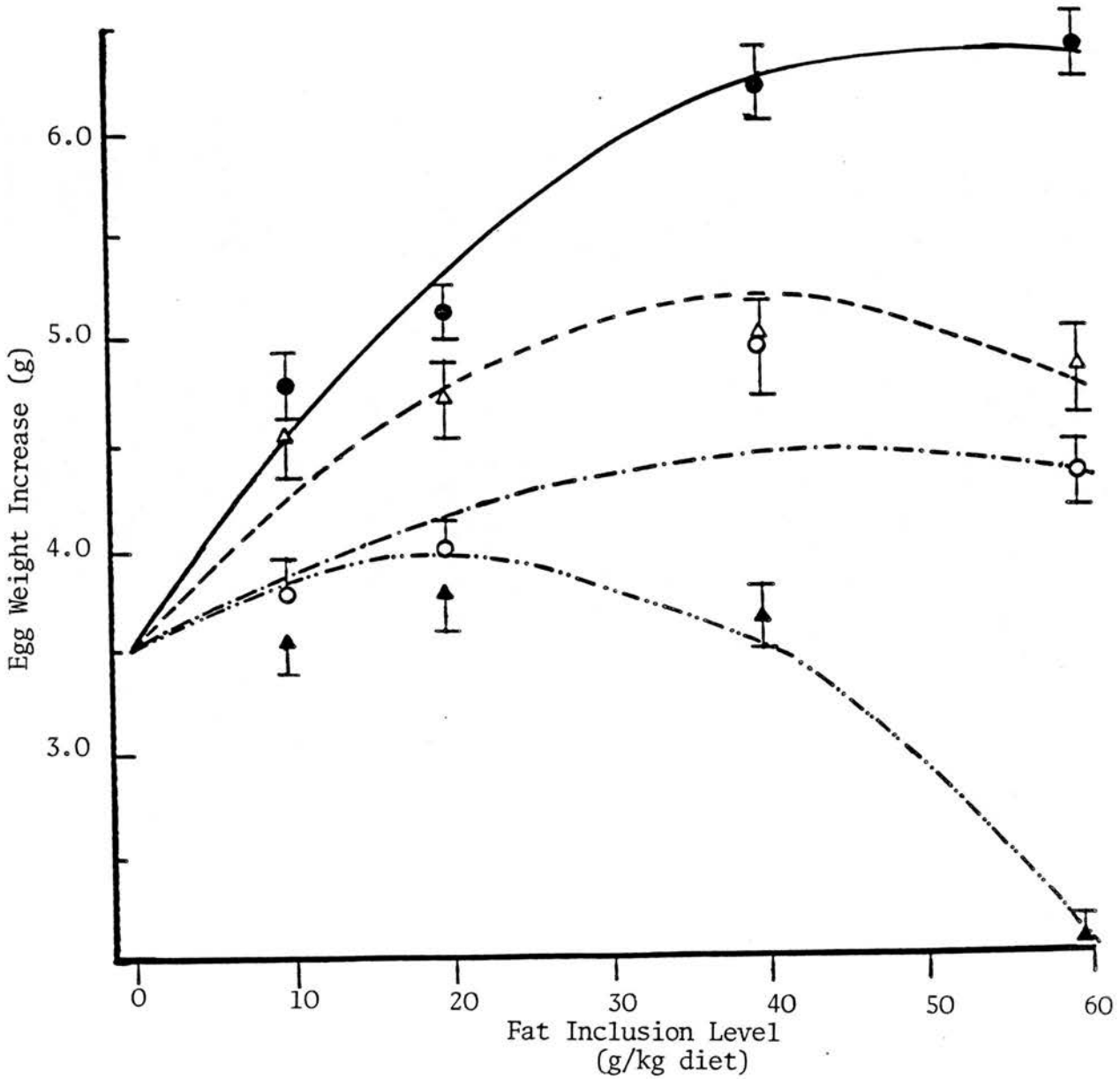


Table 5.9: The regression equations (Predictor + SD) which describe the effect of dietary fat type on egg weight

Maize	$Y = 3.46$	$+ 1.1201^{a\,1}X$	$- 0.10683^{a}X^2$
	± 0.147	± 0.1778	± 0.03104
Fish	$Y = 3.46$	$+ 0.4896^{b}X$	$- 0.11895^{a}X^2$
Tallow	$Y = 3.46$	$+ 0.8495^{c}X$	$- 0.10592^{a}X^2$
Coconut	$Y = 3.46$	$+ 0.4422^{b}X$	$- 0.04482^{b}X^2$

[1] Within columns, mean values not having similar superscripts are significantly different ($P<0.05$).

At the 20 g/kg level of inclusion, olive oil exerted a similar ($P>0.1$) increase in egg weight as maize oil. However, at the 60 g/kg inclusion level maize oil caused a significantly ($P<0.001$) greater response than olive oil.

The effect of diet on egg production during week 29 is summarised on Tables 5.10 and 5.11. Dietary treatment had a significant ($P<0.05$) though very small effect upon the rate of lay and this was determined to be due to the fat type ($P<0.001$) and not the inclusion level ($P>0.05$). However, as determined by Duncan's multiple range test (Duncan, 1955) there was no difference between the number of eggs laid by birds on the low-fat diet and any other dietary treatment except those birds fed the 60 g/kg fish oil diet ($P<0.05$). There was a significant effect ($P<0.005$) of dietary treatment on the mass of eggs laid. This is not surprising since, with the exception of fish oil at 60 g/kg, dietary treatment did not influence egg number, yet considerably affected egg weight. Two-way analysis of variance showed that whereas the inclusion level of fat did not affect ($P>0.05$) egg mass, the fat type did ($P<0.005$), and the presence of a significant ($P<0.05$) interaction suggests that the effects of these factors are not independent of each other.

The weights of internal components of eggs from birds fed the 60 g/kg inclusion level of the different fats are shown in Table 5.12. Although dietary treatment significantly ($P<0.005$) affected yolk weight, only fish oil caused a detectable ($P<0.05$) difference, namely a depression in yolk weight. Albumen weight was significantly ($P<0.05$) affected by dietary treatment, with maize oil increasing albumen deposition significantly ($P<0.05$) compared to birds fed the low-fat or

Table 5.10: The effect of dietary treatment upon weekly egg production (number of eggs + SD) after 5 weeks (week 29)

Fat Inclusion Level / Fat Type	0 g/kg (n=14)[1]	10 g/kg (n=5)	20 g/kg (n=5)	40 g/kg (n=5)	60 g/kg (n=5)
Maize Oil	6.750 ± 2.55	6.833 ± .199	6.700 ± .192	6.933 ± .109	6.679 ± .392
Fish Oil	6.750 ± .255	6.550 ± .192	6.814 ± .108	6.550 ± .183	6.182 ± .162
Tallow	6.750 ± .255	6.800 ± .162	6.836 ± .340	6.833 ± .195	6.700 ± .162
Coconut Oil	6.750 ± .255	6.711 ± .263	6.704 ± .154	6.583 ± .445	6.767 ± .149
Olive Oil	6.750 ± .255		6.733 ± .216		6.797 ± .292

	Significance of treatment effect	LSD (P=0.05)
Dietary treatment	<0.05	0.300
Fat type	<0.01	0.303
Fat level	>0.05	0.303
Type X level	>0.05	0.606

[1] Experimental group is 12 birds, i.e. N=14 is 14 groups of 12 birds

EXPERIMENTAL DETAILS

All birds were fed the low-fat diet (0g fat/kg) from 17 to 24 weeks of age. The birds were then allocated to their appropriate dietary treatment from week 25 to 29. The weekly egg production (number of eggs) was determined during week 29.

Table 5.11: The effect of dietary treatment upon the weekly egg mass produced (g + SD) after 5 weeks (week 29)

Fat Inclusion Level / Fat Type	0 g/kg (n=14)[1]	10 g/kg (n=5)	20 g/kg (n=5)	40 g/kg (n=5)	60 g/kg (n=5)
Maize Oil	390.3 ± 19.3	405.1 ± 13.6	388.5 ± 11.5	418.6 ± 7.8	401.2 ± 25.3
Fish Oil	390.3 ± 19.3	375.6 ± 14.8	389.1 ± 11.2	376.1 ± 11.6	350.5 ± 26.9
Tallow	390.3 ± 19.3	397.8 ± 10.8	403.8 ± 24.8	401.4 ± 12.3	389.9 ± 9.8
Coconut Oil	390.3 ± 19.3	382.9 ± 14.9	381.2 ± 13.2	395.7 ± 24.3	391.9 ± 11.2
Olive Oil	390.3 ± 19.3		390.8 ±10.3		395.0 ± 19.2

	Significance of treatment effect	LSD (P=0.05)
Dietary treatment	<0.005	21.2
Fat type	<0.005	20.8
Fat level	>0.05	20.8
Type X level	<0.05	41.6

[1] Experimental group is 12 birds, i.e. N=14 is 14 groups of 12 birds

EXPERIMENTAL DETAILS

All birds were fed the low-fat diet (0g fat/kg) from 17 to 24 weeks of age. The birds were then allocated to their appropriate dietary treatment from week 25 to 29. The weekly egg production (mass of eggs) was determined during week 29.

Table 5.12: The effect of dietary treatment upon the weights of the egg components and the effect of dietary fat upon the relative change in these components

Dietary Fat	N[1]	Egg Weight (g+SD)	Yolk Weight (g+ SD)	Albumen Weight (g + SD)	Shell Weight (g + SD)	Change due to dietary fat[3] Egg(g)	Yolk(g)	Albumen(g)	Shell(g)
Low Fat (0 g/kg)	7	57.51^{a}[2] ±1.34	14.44^{a} ±0.28	35.75^{a} ±1.18	7.33^{a} ±0.11	0.000	0.000	0.000	0.000
Maize Oil (60 g/kg)	5	59.97^{c} ±0.59	15.07^{a} ±0.46	37.27^{bc} ±0.31	7.64^{a} ±0.15	2.460	0.631	1.525	0.304
Fish Oil (60 g/kg)	5	56.39^{a} ±2.64	13.46^{b} ±0.42	35.74^{a} ±2.19	7.19^{a} ±0.25	-1.128	-0.981	-0.010	-0.137
Tallow (60 g/kg)	5	58.15^{b} ±1.44	14.66^{a} ±0.40	36.12^{abc} ±0.93	7.37^{a} ±0.30	0.638	0.224	0.373	0.039
Coconut Oil (60 g/kg)	5	58.31^{b} ±0.80	15.00^{a} ±0.35	35.86^{ab} ±0.67	7.45^{a} ±0.16	0.798	0.565	0.111	0.122
Significance of dietary treatment		P<0.005	P<0.005	P<0.005	P>0.05	-	-	-	-

[1] Experimental group is 12 birds, i.e. N=7 is 7 groups of 12 birds
[2] Within columns, mean values not having similar superscripts are different (P<0.05)
[3] Calculated by subtracting the mean for the low-fat treatment from the mean of the appropriate dietary treatment

EXPERIMENTAL DETAILS

All birds were fed the low-fat diet (0g fat/kg) from 17 to 24 weeks of age. The birds were then allocated to their appropriate dietary treatment from week 25 to 29. Eggs were collected on 2 consecutive days during week 29. The eggs were stored at 4 C and the yolk,albumen and shell weights from one egg per bird were determined within 10 days of collection.

fish oil diet. Dietary treatment did not influence ($P>0.05$) shell weight.

An estimate of the change in the egg components due to the presence of 60 g/kg of the different dietary fats was calculated by subtracting the mean for the low-fat data from that of the mean for the appropriate fat (Table 5.12). These calculated figures, though unsuitable for statistical analysis (due to lack of replication), do suggest that there may be differences in the relative importance of the yolk or albumen in the mechanisms by which the different dietary fats affect egg weight in birds of this age. Fish oil apparently causes a reduction in egg weight by depressing the yolk size whilst having no effect upon albumen weight. It is of interest to note that whilst maize and coconut oils increased yolk weight to the same degree, maize oil increased albumen weight 13-fold more than coconut oil. Also, it can be seen that tallow caused an increase in yolk weight only half as much as did coconut oil, yet stimulated an increase in albumen weight which was three times that of coconut oil.

The investigation of the egg components from birds fed the graded levels of maize oil (Table 5.13) demonstrated some strong relationships. There were significant correlations between the level of maize oil present in the diet and egg weight ($P<0.001$), yolk weight ($P<0.01$) and albumen weight ($P<0.001$), but no such correlation was found for shell weight ($P>0.1$).

No significant ($P>0.1$) effect of dietary treatment upon plasma oestradiol concentration could be detected, nor could the means (Table 5.14) be separated at the 5% probability level by Duncan's multiple range test (Duncan, 1955). However, a comparison (Fig 5.2) of the data

Table 5.13: The relationship between the weight of egg components (mean + SD) and the inclusion level of maize oil in the diets

Egg with Component	Maize oil inclusion level					Correlation
	0 g/kg	10 g/kg	20 g/kg	40 g/kg	60 g/kg	inclusion level
Egg wt(g)	57.51±1.34	57.71±1.09	58.11±0.54	60.48±1.43	59.97±0.60	0.704**
Yolk wt(g)	14.44±0.28	14.58±0.74	14.77±0.40	15.26±0.31	15.07±0.46	0.535*
Albumen wt(g)	35.75±1.18	35.16±0.94	35.80±0.51	37.44±1.06	37.27±0.31	0.635**
Shell wt(g)	7.33±0.11	7.96±0.62	7.54±0.17	7.78±0.36	7.64±0.15	0.190

* P>0.01; ** P>0.001

EXPERIMENTAL DETAILS

All birds were fed the low-fat diet (0g fat/kg) from 17 to 24 weeks of age. The birds were then allocated to their appropriate dietary treatment from week 25 to 29. Eggs were collected on 2 consecutive days during week 29. The eggs were stored at 4°C and the yolk,albumen and shell weights from one egg per bird were determined within 10 days of collection.

Table 5.14: The effect of dietary fat type and inclusion level on concentration of circulating oestradiol

Dietary fat and inclusion level (g/kg)	N[2]	Oestradiol concentration (pg/ml plasma + SEM)
Low Fat (0 g/kg)	25	314.6[a 1] ±20.0
Maize Oil (20 g/kg)	28	364.3[a] ±24.5
Maize Oil (40 g/kg)	23	382.7[a] ±32.7
Maize Oil (60 g/kg)	24	386.1[a] ±32.0
Fish Oil (20 g/kg)	25	328.6[a] ±23.1
Fish Oil (60 g/kg)	15	297.1[a] ±18.2
Tallow (20 g/kg)	20	371.3[a] ±30.8
Tallow (60 g/kg)	24	360.7[a] ±27.0
Coconut Oil (60 g/kg)	22	341.0[a] ±26.2
Olive Oil (60 g/kg)	20	348.5[a] ±35.6

Significance of treatment, P>0.1

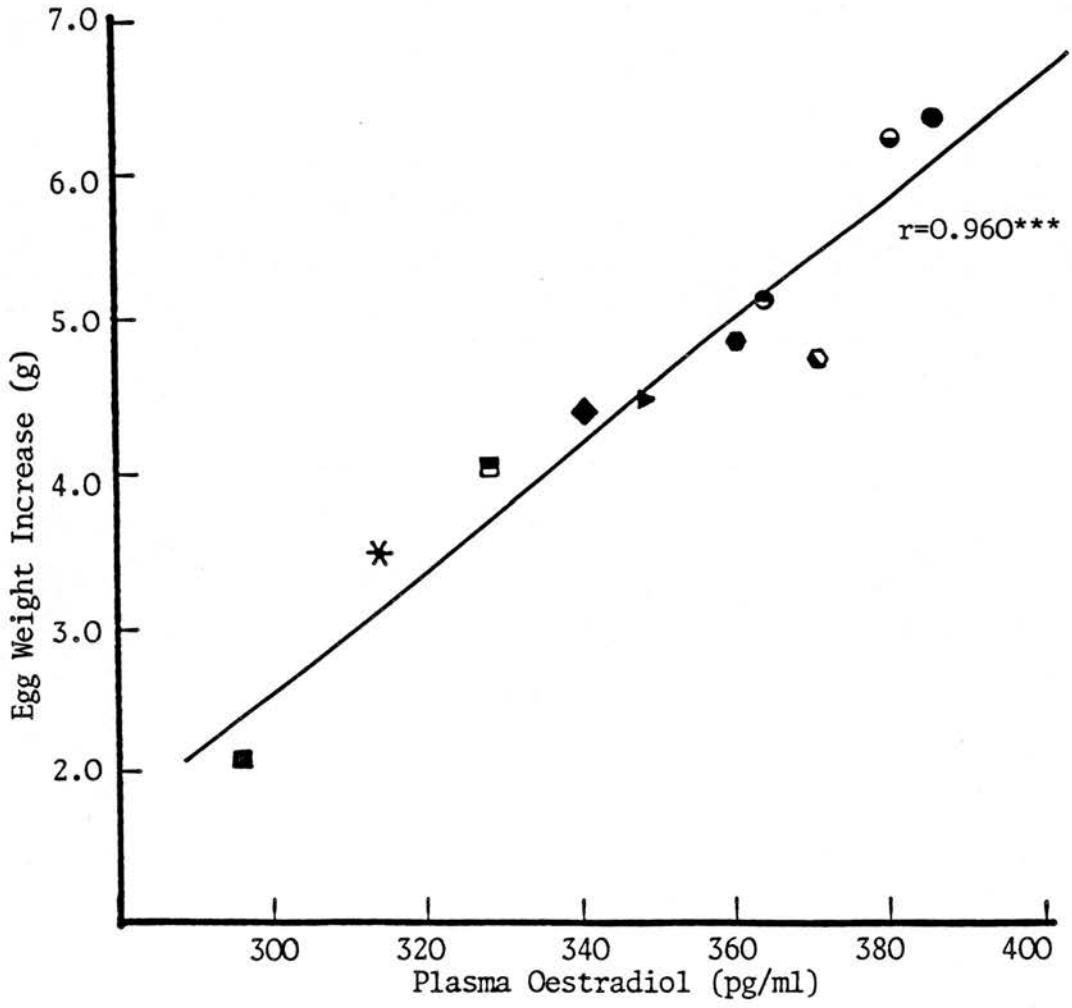
[1] Within columns, means values not having similar superscripts are different (P<0.05)
[2] N = Number of birds

EXPERIMENTAL DETAILS

All birds were fed the low-fat diet (0g fat/kg) from 17 to 24 weeks of age. The birds were then allocated to their appropriate dietary treatment from week 25 to 29. Blood samples were obtained between 08.00-10.00 hrs on one day during week 29. Only birds that were noted to have laid an egg on the day of the blood sampling and on the preceding and following day were included in the oestrogen determination. Oestradiol-17β was measured by a double antibody radioimmunoassay following an initial purification (see Chapter 2).

Fig 5.2:The relationship between plasma oestradiol concentration and the increase in mean egg weight of hens fed different fat types and contents (see below for key to symbols) over the 5-week experimental period.

(***,correlation coefficient significant at 0.1% level of probability)



INCLUSION LEVEL (g/kg diet)	FAT TYPE				
	Maize Oil	Fish Oil	Tallow	Coconut Oil	Olive Oil
0	✳	✳	✳	✳	✳
10	○	□	○	◇	
20	◒	▣	◐	◆	▷
40	◒	▣	◐	◇	
60	●	■	●	◆	▶

for the change in egg weight due to the dietary treatments and the corresponding mean oestradiol concentrations showed a linear relationship with an extremely high degree of correlation ($r=0.960$, $P<0.001$). Significant correlations also existed between the plasma oestradiol concentration and the yolk weight ($r=0.862$, $P<0.02$) and the albumen weight ($r=0.774$, $p<0.05$).

IV. DISCUSSION

By formulating carefully the diets to contain very similar ME and protein contents, the present experiment allowed a thorough investigation into the effect of a variety of dietary fats fed at several inclusion levels on the performance of laying hens and gave some indication of the mechanism by which the changes might have been brought about.

Feed consumption was unaffected by changes in the dietary fat content, as found by many other workers (e.g. Karunajeewa and Tham, 1987; Scragg et al., 1987 and Whitehead, 1981). However, there was a slight reduction in food intake by birds fed the high level of fish oil (60 g/kg). Other workers have not reported any effect of fish oil on feed consumption (Marion and Edwards, 1964; Menge et al., 1965a and 1965b; Balnave, 1970b) with the exception of Balnave (1967) who found that fish oil ethyl esters caused such a drastic reduction in food intake that the birds ceased to lay. However, this was probably due to the instability of the polyunsaturated fatty acid ethyl esters used and comparisons with the more stable glyceride form of the fish oils used in the present experiment may be inappropriate.

Many of the published reports on the effects of different dietary fat types on egg weight are contradictory and it was hoped that the present experiment would provide not only more reliable data, but also an indication as to why previous results have been so variable. It is clear that all of the fat types exerted a significant effect upon egg weight but it can be seen from the response curves (Fig. 5.1) that inadequate experimental designs could fail to detect changes in egg weight. The type of experimental design and the method of data analysis can be important in understanding the responses of hens to dietary fat. The feeding of several levels of a fat and the use of response curve analysis allows a more sensitive statistical comparison than is possible when only two treatments are compared, especially when only small differences in egg weight are involved. Thus, the use of the latter approach has resulted in other authors reporting no effect on egg weight of tallow fed at levels of 30 g/kg (Jensen et al., 1958) and 25 g/kg (Treat et al., 1960) or cod liver oil fed at 20 g/kg (Balnave, 1970b).

All of the fat types investigated significantly affected egg weight in a manner that could be adequately described by a quadratic function that was unique to the individual fat type. In each case the optimal dietary fat content was less than the highest inclusion level (60 g/kg) and varied from approximately 21 g/kg for fish oil to 52 g/kg for maize oil. Extrapolation from the calculated response curves to expected changes in egg weight at higher levels is ill-advised as major changes in diet composition would be required to maintain similar nutrient compositions and may change many aspects of the laying hen's performance. However, the report that both 100 g/kg tallow and maize

oil significantly increase egg weight (Combs and Helbacka, 1960) is difficult to accept unless a model different from a quadratic function is required to explain the effect of some dietary fats over a greater range of inclusion levels.

Though the diets used in the present experiment were formulated to be adequate in all known nutrients, some of the diets were marginally deficient in their linoleic acid content which were slightly less than the recommended requirement of 9 g/kg (Whitehead, 1981). However, having been reared on a linoleic acid-adequate diet to the age of 17 weeks it can be assumed that the bird's adipose tissue reserves would ensure no deleterious effect of the slightly linoleic acid deficient diets which were only fed for a short period. The difficulty of inducing linoleic acid deficiency in adult hens was discussed in Chapter 1.

Perhaps the area in which the greatest controversy exists about the effect of dietary fat upon egg weight is whether linoleic acid has a greater influence upon egg size than other readily absorbable dietary fats (see Chapter 1). It has been suggested (Shannon and Whitehead, 1974) that a source of readily absorbable monounsaturated fatty acids (e.g. oleic acid) are equally effective as linoleic acid in eliciting a response in egg weight. Conversely, Scragg et al. (1987) suggested that increasing the intake of readily absorbable fat without increasing the linoleic acid intake does not increase egg weight. The present experiment allowed a comparison between the relative potencies of maize oil (56% linoleic acid) and olive oil (7% linoleic acid). Maize oil was far more effective in causing egg weight to increase than olive oil, though olive oil did significantly increase egg weight. In the

present experiment, dietary fats higher in linoleic acid content gave larger increases in egg weight, but this was not necessarily true of the dietary treatments themselves. In addition, the quadratic nature of the response curves for all of the fat types tested indicate that there must be factors other than merely the linoleic acid content of the diet which influence the change in egg weight. It seems likely that increased linoleic acid intake is not necessary for a response in egg weight though it would appear that dietary fats high in linoleic acid are the most effective. This is further discussed in Chapter 6.

The present experiment was designed to investigate the relationship between the chain length and degree of saturation of the fatty acids of the different fat types and the subsequent response in egg weight. Fish oil contained the longest chain fatty acids and the highest degree of unsaturation, coconut oil was the opposite, whilst maize oil and tallow were intermediate. Maize oil was the most effective in increasing egg weight and coconut oil was least effective with tallow intermediate and fish oil depressing egg weight. These findings would indicate that extremes of fatty acid chain length and of the degree of saturation result in poor increases in egg weight, whereas medium chain fatty acids elicit large increases in egg weight. It would appear that if a relationship exists between the response in egg weight and the fatty acid chain length and degree of saturation it is not of a simple nature.

An insight into the mechanism by which the dietary fats affect egg weight is given by the consideration of the change in the internal components of the egg. Maize oil, at an inclusion level of 55 g/kg was shown to increase both yolk and albumen in young birds (Chapter 3) and

this was found to be repeated in the present experiment with an increase in both yolk and albumen weight associated with increasing inclusion levels of maize oil. However, the different relative changes in the internal components of eggs from birds fed different fat types suggest that these changes in yolk and albumen weight depend upon the fat type. This conclusion, taken along with the observation that the change in internal components due to dietary fats is age-dependant (see Chapter 3), might account for the varied reports published on the response of egg components to dietary fat.

The determined levels of circulating oestradiol described in Chapter 4 for birds on the control and 55 g/kg maize oil diet were approximately only 77% and 82%, respectively, of the levels in the present experiment. The higher concentrations of oestradiol detected in the present experiment may have been due to: slight differences in the diet compositions; the ages of the birds; or because the determinations were performed within weeks of plasma collection rather than months. There seemed to be large variances associated with the oestradiol determinations from each of the dietary treatments, and the coefficient of variation was approximately 36%. Many workers have reported similar large variations in the determined oestradiol levels in laying hen plasma with coefficients of variation, of 54% (Shodono et al., 1975); 49% (Hagan et al., 1984) and 47% (Leszczynski et al., 1984). The dietary treatments did appear to affect the oestradiol levels but due to the large variances, this effect was not statistically significant. The extremely high correlation (r=0.960) between the group means for the oestradiol concentrations and the increases in egg weight strongly suggests that dietary fat brings about

its effect on egg weight via its effect upon oestradiol levels. It was discussed in Chapter 4 how increased oestradiol levels might influence yolk deposition by providing more yolk precursors, and affect albumen weight by stimulating the oviduct to synthesize more albumen proteins. The effect of different dietary fats upon oestradiol levels appears to have received very little attention in any experimental model, and possible mechanisms are discussed more fully in Chapter 6.

CHAPTER 6

DISCUSSION

The suggestion that dietary manipulation can increase egg weight by a maximum of 1 gram (Morris, 1985) has been shown to be a considerable underestimation. Indeed, the 2.9 gram increase in egg weight obtained in this study, which would be worth approximately a further £30M annually to the UK egg producers (calculated from Anon, 1988) clearly indicates the improvements that further poultry nutrition research may achieve. Research into important nutritional phenomena, such as the effect of dietary fat upon egg weight, benefit from complementary investigations at various levels. In this study the role of dietary fat in egg weight has been examined at the gross level by investigating the whole egg and its internal components (Chapter 3) at the flock level (Chapter 5), and using a biochemical approach to elucidate the physiological basis of the observed effects (Chapter 4).

A diet containing maize oil at an inclusion level of 55 g/kg was used to establish a suitable experimental model to investigate the effect of "dietary fat" upon egg size initially at the gross level (Chapter 3) and then biochemically (Chapter 4). In response to feeding the 55 g/kg maize oil diet the egg weight increase was rapid, but no further effect of dietary fat upon egg weight was detectable after approximately 20 days. The potential for dietary fat to increase egg weight appeared to diminish with the age of the hen. Yolk weight was only affected in young birds, though albumen weight responded to fat-feeding regardless of age. The age-dependent manner by which dietary fat affects yolk weight might be explained by accepting the idea that yolk and albumen have theoretical maximum weights (Jenkins

and Taylor, 1960) and that the maximum yolk weight is reached at a relatively early age after which dietary fat can no longer exert a detectable effect. This limitation is not so apparent for albumen weight.

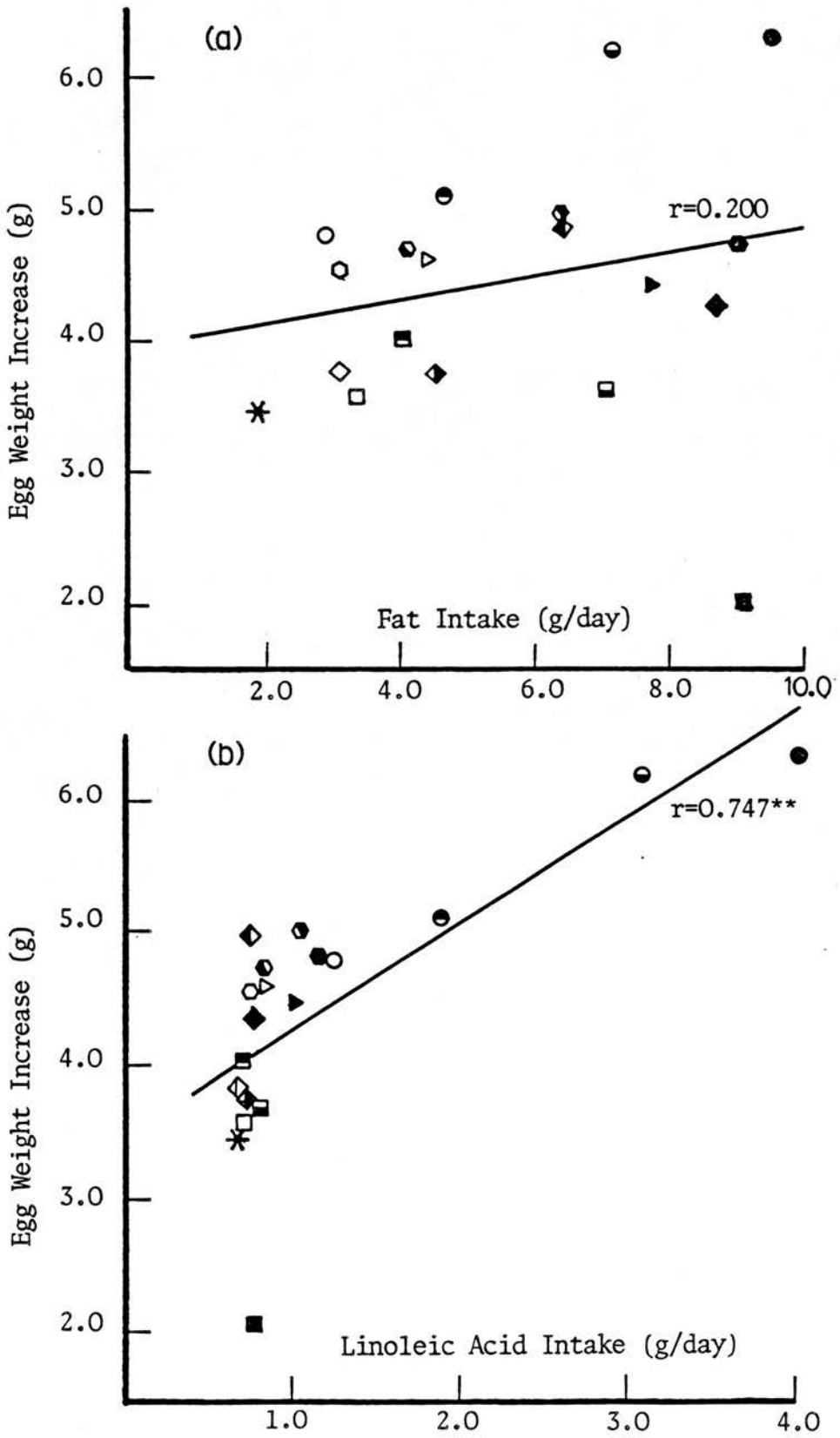
The present study demonstrated that dietary fat induces rapid responses in egg weight and an equally rapid reduction in egg weight resulted from the transfer of the hen onto a low-fat diet (Chapter 3). The ability to increase or reduce egg weight in this way has been suggested to be of significance to egg marketing authorities which increasingly are attempting to balance egg output to consumer demand (Balnave, 1987). Increasing egg weight is especially important for hens just coming into lay when large numbers of eggs are too small to be sold for whole-egg consumption. In addition, with the hatchability of eggs from young birds being as low as 40% in the broiler breeder industry (Noble et al., 1984), the increased egg weight due to dietary fat and the subsequent increased embryonic size and hatchability (Shanawany, 1985) would be of commercial importance. Lastly, dietary fats may be useful in maintaining egg weights when hens are kept at ambient temperatures that are high enough to reduce feed consumption and cause an energy and protein deficiency, as suggested by Sell et al. (1987) and Balnave (1987). This dietary manipulation would be particularly advantageous in a flock where the early period of lay coincides with the hot season (Pell and Polkinghorne, 1986).

The relative potencies of different fat types fed at various levels upon egg weight were investigated in Chapter 5. Maize oil caused the greatest increase in egg weight and coconut oil the least, with tallow being intermediate. In contrast, high levels of dietary fish oil

depressed egg weight. The optimum inclusion level for all of the fats tested was less than 60 g/kg. Excepting fish oil, the number of eggs produced was not affected by the dietary fats whilst the mass of eggs was significantly increased. The dietary treatments had no effect upon food consumption. Hence, with the exception of fish oil, incorporation of fats into layer diets resulted in improved feed conversion efficiency and an increase in the number of eggs in the higher weight categories in which eggs are sold.

Explanations for the differing potencies of the fats tested are not obvious. It would seem that the response of egg weight to dietary fat is not simply dependent upon the fatty acid chain length or the degree of saturation. The questions of whether linoleic acid has a greater influence upon egg size than other readily absorbable dietary fats was discussed in detail in Chapter 1. After reviewing the literature the present author concluded that while dietary fats high in linoleic acid generally resulted in larger increases in egg weight, fats low in linoleic acid could also increase egg weight. The large-scale nutrition experiment described in Chapter 5 allowed a further investigation of this area. Dietary fats which were high in linoleic acid gave superior increases in egg weight, but this was not necessarily true of the dietary treatments themselves. The relationships between egg weight and both daily fat intake and daily linoleic acid intake are shown in Fig 6.1. It would appear that since egg weight is significantly correlated ($r=0.747$, $P<0.01$) with daily linoleic acid intake, but not ($r=0.200$, $P>0.05$) with daily fat intake, that linoleic acid is the cause of dietary fats' effect upon egg weight. However, care is needed to interpret these results. If the

Fig 6.1:The relationship between the increase in egg weight over the 5-week experimental period and (a) daily fat intake and (b) daily linoleic acid intake in hens fed different fat types and amounts (see opposite for key to symbols). (**,correlation coefficient significant at 1% level of probability)



data for the two highest inclusion levels of maize oil are omitted from the analysis, then the correlation between egg weight and daily linoleic acid intake ceases to attain statistical significance. In addition, it should be noted that since high levels of fish oil depress egg weight, there can be no simple relationship between dietary fat and egg weight. Indeed, the fact that dietary fats affect egg weight in a quadratic manner and that fish oil depressed egg weight would indicate that there must be factors other than the linoleic acid content involved.

Information gained during this study will be of interest to the animal-feed industry and will be useful in the formulation of layer diets. Though the exact importance of linoleic acid in the effect of dietary fat upon egg weight is uncertain, it would appear that, in general, fats high in linoleic acid are the most potent. Cheaper alternative feedstuffs should be sought which possess the properties that the potent dietary fats, such as maize oil, contain (perhaps high linoleic acid contents). In such a way, rice pollard, with its high linoleic acid content, has been shown to elicit the same egg weight response as vegetable oils (Balnave, 1982).

Relatively large-scale nutritional studies involving careful experimental design and data analysis are necessary to detect the small changes in yolk and albumen weight which at best are <1.5% and <2.5% of the egg weight, respectively. For this reason, studies at the biochemical level to elucidate the mechanism by which dietary fat affects egg weight (Chapter 4), which employ greatly reduced numbers of replication and are targeted at more specific aspects of egg formation, are more difficult to secure definitive statements.

An attempt to explain how dietary fat increases yolk and albumen weight was made by investigating the metabolism of triglyceride-rich lipoproteins. Maize oil, at 55 g/kg, caused a slight increase in the plasma levels of the yolk precursors, VLDL; this was despite a three-fold increase in the post-heparin lipase activity, which is responsible for the hydrolysis of VLDL triglyceride. The oviducal tissues, responsible for the albumen protein synthesis, possessed considerable Lpl activities and removed significant quantities of triglyceride from the circulation, notably VLDL, reflecting the importance of triglyceride-rich lipoproteins in the metabolism of the oviduct. These findings suggest that raised plasma levels of VLDL may be part of the mechanism by which dietary fat increases yolk and albumen weight.

The results on plasma oestradiol concentrations obtained in the present study gave the best indication for the more specific biochemical basis of the mechanism by which dietary fats affect egg weight. The findings that 55 g/kg maize oil significantly ($P<0.01$) increases levels of circulating oestradiol and that there is a high correlation ($r=0.960$) between the change in egg weight and the mean plasma oestradiol concentrations in birds fed a variety of fat types and inclusion levels would indicate that dietary fats increase egg weight via their influence upon oestradiol metabolism.

There are several possible mechanisms by which oestrogen might influence egg weight. The higher concentration of VLDL in birds fed the 55 g/kg maize oil could be a consequence of the oestrogen stimulated increase in hepatic triglyceride synthesis (Kudzama, et al., 1975), phospholipid biosynthesis (Vigo and Vance, 1981) and

VLDL-apolipoprotein synthesis (Wiskocil et al., 1980). Oestrogen is required to achieve and maintain the mature size of the oviduct and albumen protein synthesis (Schimke et al., 1973 and Okulicz et al., 1985), and has been shown to induce its own receptors in the chick oviduct (Sutherland and Baulieu, 1976). Presumably dietary fat causes an increase in albumen weight via an increase in circulating oestradiol, though there is no evidence in laying hens that additional oestrogen has this effect. There appears to be no effect of age upon the oestrogen levels in the hen (Okulicz et al., 1985). Hence, the age-dependent nature of yolks response to dietary fat cannot easily be explained with reference to oestrogen metabolism.

The observation that dietary fat causes a three-fold increase in post-heparin lipase activity (PHLA), though supported by experimental evidence in humans (Fredrickson et al., 1963 and Porte et al., 1966), is contradictory to the numerous reports on the effect of oestrogen upon PHLA. Oestrogen treatment has been shown to greatly depress PHLA in humans (Hazzard et al., 1969 and Applebaum et al., 1977), rats (Kim and Kalkhoff, 1975) and turkeys (Kelly et al., 1976). It is difficult to reconcile the conflicting findings that dietary fat increases both PHLA and oestrogen levels other than to highlight the complexity of this experimental model. Oestrogen levels were affected by the diets fed to the hens which can be considered as high- and low-fat diets or, alternatively, low- and high-carbohydrate diets. Hence, the resultant PHLA is due to the balance of insulin, glucagon, gluco-corticoids, oestrogen and other factors. Indeed, working with rats, Valette et al. (1987) reported that the response of Lpl activity to oestrogens is more complex than previously thought, being dependent

upon the type, dose and duration of oestrogen treatment in addition to the nutritional state of the animal.

Though maize oil, olive oil, coconut oil and tallow raised plasma oestrogen levels relative to a low-fat diet, high levels of fish oil tended to have the opposite effect. The mechanism by which different dietary fats exert their effects upon oestrogen levels in the laying hen cannot be adequately explained without further investigation, but some of the alternatives are discussed below.

One possible explanation for the increase in plasma oestrogen levels is the presence of plant compounds with oestrogenic activity, phytoestrogens (for review see Livingston, 1978), in the fats studied, except for tallow and fish oil. However, this notion is unlikely for several reasons. Firstly, levels of phytoestrogens capable of exerting biological effects are usually associated with forage crops, not the plants from which these fats were extracted. Secondly, phytoestrogens have been reported to greatly reduce the number of eggs laid in turkeys (Leopold et al., 1976) and Bobwhite quail (Lien et al., 1987). No effect on egg production was found in the present study with dietary treatment (see Chapters 3 and 5). If dietary fats can therefore be discounted as sources of exogenous oestrogens, it would appear that they may influence oestrogen levels via an effect upon oestrogen metabolism.

It is conceivable that dietary fat may stimulate oestrogen synthesis by increasing the amount of available plasma cholesterol, possibly in the form of VLDL and portomicron-remnants. All steroidogenic cells have relatively large requirements for cholesterol and this is true for the granulosa and thecal cells of the hen ovary which are

stimulated to synthesise progesterone and oestrogen respectively, by gonadotrophins (see Wells and Gilbert, 1984). Though steroidogenic cells are capable of synthesising cholesterol, the obligate intermediate in steroid hormone production, uptake of lipoprotein cholesterol is the principal means by which the adrenals, ovaries, placenta and testes acquire substrate for steroidogenesis in several species (for review see Gwynne and Straus, 1982). In most species the LDL fraction is the major source of cholesterol for a variety of steroidogenic tissues. At the onset of egg production the distinction between LDL and VLDL in the hen becomes less clear, their sizes overlap to some extent (Chapman et al., 1977) and their lipid compositions become more alike (Chapman, 1980). Therefore, VLDL may provide the cholesterol for steroidogenesis in the ovary. Alternatively, portomicron-remnants may be a source of cholesterol. Anderson and Dietschy (1977a) showed that though chylomicrons do not provide cholesterol for steroidogenesis in the rat adrenals and ovary, the smaller chylomicron-remnants can (Anderson and Dietschy, 1977b).

A detailed account of ovarian morphology is given by Gilbert and Wells (1984) and only comments relevant to the effect of dietary fat on steroidogenesis will be discussed here. The oocyte, containing the yolk material, is surrounded by several layers of different cell types (Plate 6.1). Between the perivitelline layer and the basal lamina is the one-cell thick granulosa layer which is responsible for the conversion of cholesterol to progesterone. Thecal cells are incapable of synthesising oestrogen de novo but are known to convert the progesterone diffusing from the granulosa cells into oestrogen (Huang et al., 1979). Hence, dietary fat can only increase oestrogen

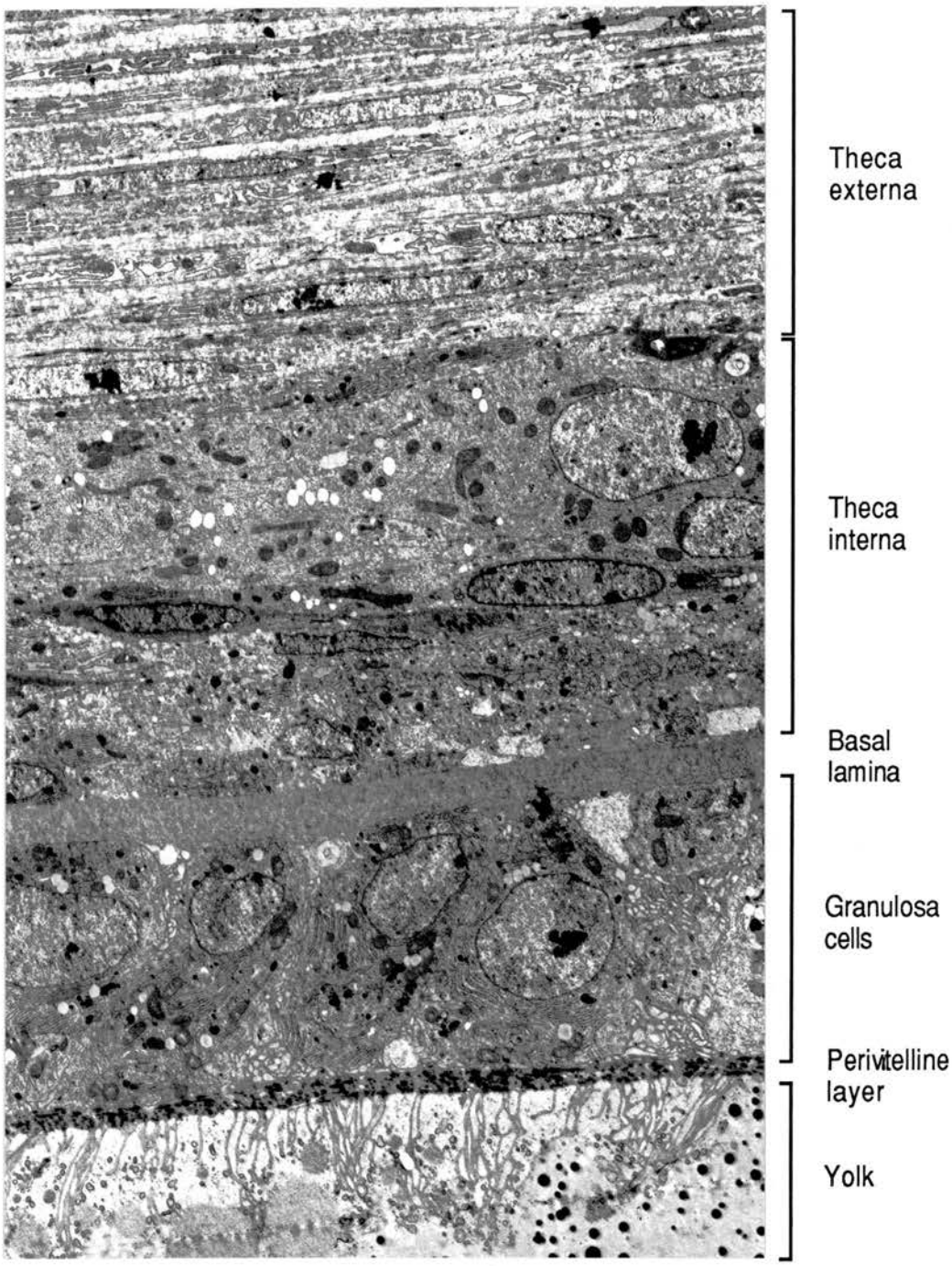


Plate 6.1

Electron micrograph of a section through a follicle to illustrate thevarious components of the wall. The follicles were taken from 29 week old hens fed the 50g/kg maize oil diet (see table 3.1 for composition) and prepared for transmission electron microscopy as described by Griffin and Perry (1985). (Mag. x 3648).

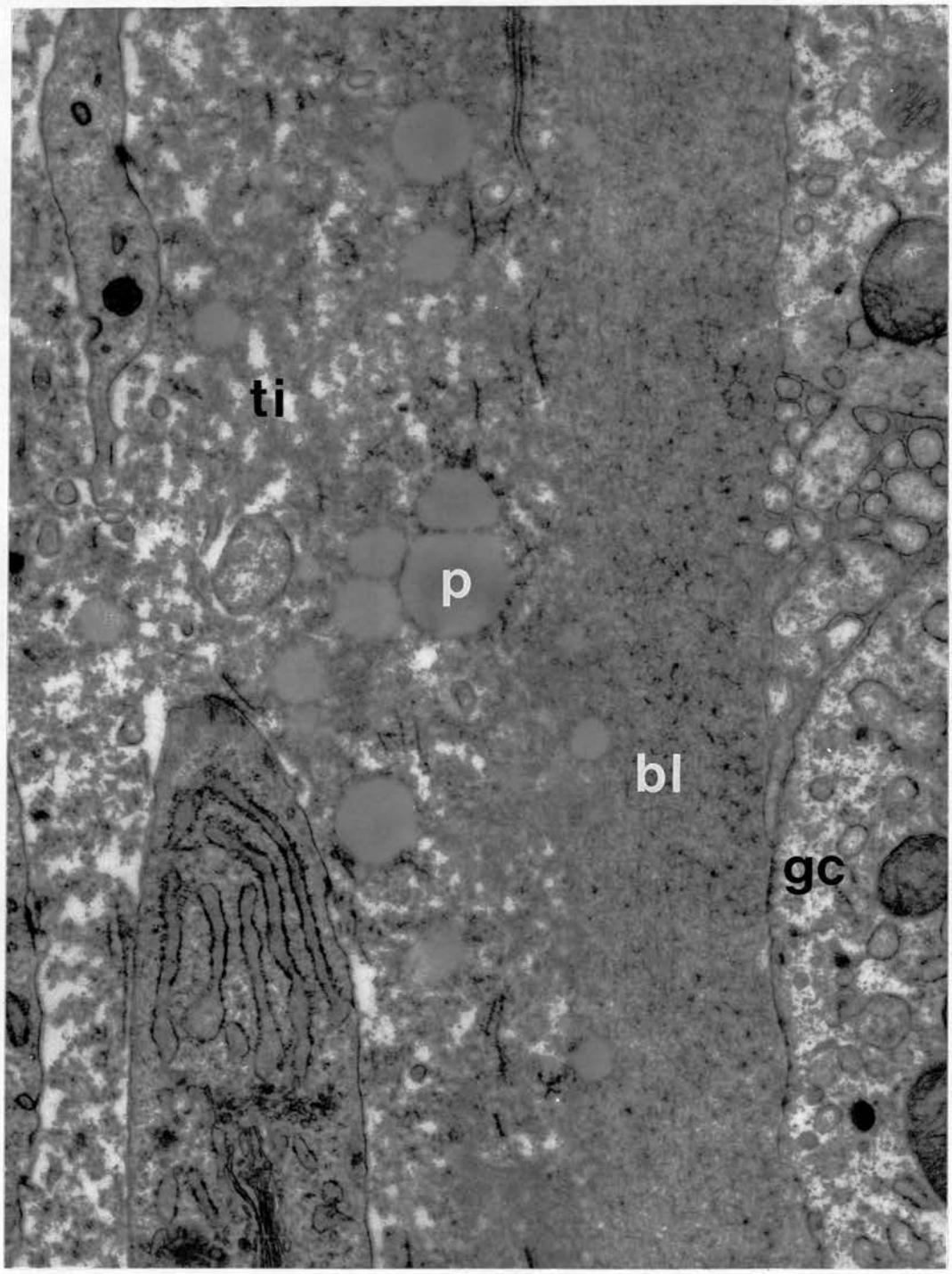


Plate 6.2

Electron micrograph of the follicle wall near the basal lamina from a bird fed on the high-fat diet (50g/kg maize oil). Note the accumulation of portomicrons (P) on the boundary of the basal lamina (bl) which separates the theca interna (ti) and the granulosa cells (gc). Practical details are as for plate 6.1. (Mag × 25,056)

levels via progesterone synthesis by supplying extra cholesterol to the granulosa cells. As discussed earlier (Chapter 4), nascent portomicrons can not pass through the basal lamina due to their large size but accummulate at its outer boundary in birds fed high-fat diets (Plate 6.2 and Griffin and Perry, 1985). However, smaller portomicron-remnants which increase in the circulation with maize oil feeding (Chapter 4) may be able to reach the granulosa cells and provide an exogenous source of cholesterol. Indeed, the finding that follicle membranes contained a significant amount of radioactive label which was originally injected as newly synthesised nascent portomicrons (Chapter 4) might support this idea.

The supposition that fat-feeding raises plasma oestrogen levels by providing increased cholesterol in the form of VLDL or portomicron-remnants for oestrogen synthesis fails to explain all of the observations. It is generally considered that dietary saturated fatty acids increase both lipoprotein and plasma cholesterol more than unsaturated fatty acids, yet maize oil caused the greatest increase in oestrogen levels. In addition, fish oil depressed oestrogen levels. These considerations suggest there are other mechanisms by which dietary fats affect oestrogen metabolism other than simply providing more cholesterol, perhaps by altering the enzymatic degradation and synthesis of oestrogen.

The initial step in the degradation of the major oestrogens in the hen is a hydroxylation reaction in the liver (Schneider et al., 1983) involving the mixed function oxidase enzyme system in the microsomal membrane (see Yang, 1977 and Paine, 1981). This liver microsomal enzyme system, which is responsible for the biotrans-

formation of steroids, fatty acids, drugs, carcinogens and other xenobiotics, can be resolved into three components: cytochrome P-450, NADPH-cytochrome P-450 reductase and phospholipid (Coon, 1978). The function of the phospholipids, which make up more than 25% (w/w) of the microsomal membrane (Yang, 1977), is to form a fluid environment for electron transport and to hold cytochrome P-450 in an active conformation (Ingleman-Sundberg, 1977). Microsomal enzyme activity is known to be greatly increased by treatment with a variety of drugs, such as phenobarbital, and this response involves enhanced phospholipid synthesis (Conney, 1967). There appears to be a requirement for dietary lipid in the drug induced increase in the microsomal enzyme system activity (Century and Horwitt, 1968; Marshall and Maclean, 1971; and Hammer and Wills, 1979). Diets rich in unsaturated fats allow the greatest increase in enzyme activity, especially fish oil (Century and Horwitt, 1968), though Hammer and Wills (1979) showed that destruction of the polyunsaturated fatty acids in fish oil by irradiation caused fish oil to lose this potency. It is possible that the depression in plasma oestrogen levels observed in hens fed diets containing fish oil was due to an increased rate in their degradation due to the hepatic microsomal phospholipid incorporation of unsaturated fatty acids, especially of the n-3 type (Hammer and Wills, 1979). Supporting this theory is the report that the plasma oestradiol levels in young male chicks with oestrogen implants were lower in birds fed diets containing fish meal, relative to diets containing maize meal (Akiba et al., 1982). Subsequent work by Takahashi and Jensen (1984) revealed that the fish meal diet increased both the rate of oestrogen degradation and the activity of the hepatic microsomal enzyme system. However, this

theory does not explain all of the observations, as maize oil, being rich in unsaturated fatty acids, though of a shorter chain length, increased the plasma oestrogen concentrations.

The synthesis of progesterone from cholesterol by the microsomal fraction of the ovarian granulosa cells (Talalay, 1965) and the subsequent conversion to oestrogen by the microsomal fraction of the thecal cells (Armstrong and Wells, 1976) requires similar enzyme systems as that involved in the hepatic degradation of oestrogen. The role that dietary fats, especially those rich in unsaturated fatty acids, play in increasing the activity of the hepatic microsomal enzyme system is likely to occur in the ovarian microsomes, resulting in increased oestrogen synthesis. The observation that the unsaturated fats, maize oil and fish oil, have opposite effects on oestrogen levels might be reconciled by the suggestion that different cytochrome P-450 species have different specificities for the fatty acid moieties of the phospholipids stabilising the enzyme in an active conformation (Ingleman-Sundberg and Gustafsson, 1975).

It would seem that the effect of dietary fats upon oestrogen metabolism may be dependent upon the properties of the specific fatty acids of the fats rather than simply their degree of saturation. The effect of a dietary fat upon plasma oestrogen levels is a function of its ability to stimulate both the synthesis and the degradation of oestrogen in the ovary and liver, respectively.

It is apparent that on the limited experimental evidence obtained, no conclusive statements can be made about the mechanism by which dietary fats affect plasma oestrogen levels, and the above discussions are only postulations requiring much further study. As far

as the author is aware, the finding that dietary fat affects oestrogen is unreported and much further investigations are required in this area. It would be of interest to measure the effect of dietary fat upon oestrogen levels through the entire oestrus cycle to see whether oestrogen is raised throughout the cycle or only when measured in this study (approximately 3 hours pre-ovulation). In addition, since progesterone and testosterone are precursors in oestrogen synthesis, the plasma concentrations of these hormones might also increase. The possibility that dietary fats may increase levels of circulating sex hormones, which are known to stimulate lean-growth in many livestock species, could be of considerable commercial importance. This is especially true since the use of synthetic anabolic steroids, such as trenbolane acetate and stilbosterol, has recently become illegal.

The effect of dietary fats upon the in vivo synthesis and degradation of oestrogen could be determined by injecting labelled cholesterol and oestradiol, respectively. However, great care would be required to ensure that any effect of dietary fat upon oestrogen metabolism was not masked by the position of the hen within its oestrus cycle. This problem could be eliminated using cultured granulosa and thecal cells, separately and in combination, to study the in vitro steroidogenesis using substrates such as lipoproteins from different dietary treatments, or using labelled precursors such as cholesterol, pregnenolene and progesterone. Equally, oestrogen degradation could be studied in vitro using cultured hepatocytes.

In summary it would appear that the change in egg weight observed when different fat types are fed is associated with the resultant plasma oestrogen concentrations. Mechanisms by which dietary

fats can affect the synthesis of the yolk and albumen components via oestrogen are supported by experimental or cited evidence, but the mechanism by which dietary fats might affect the plasma oestrogen concentrations are much less clear. The influence of dietary fat upon sex steroid hormone levels being a very important, and hitherto uninvestigated issue, it is suggested that this area warrants much further study both in the medical and agricultural context. In addition, the present study provides much practical information which could be of interest for the diet formulation and husbandry of laying hens.

APPENDIX

APPENDIX

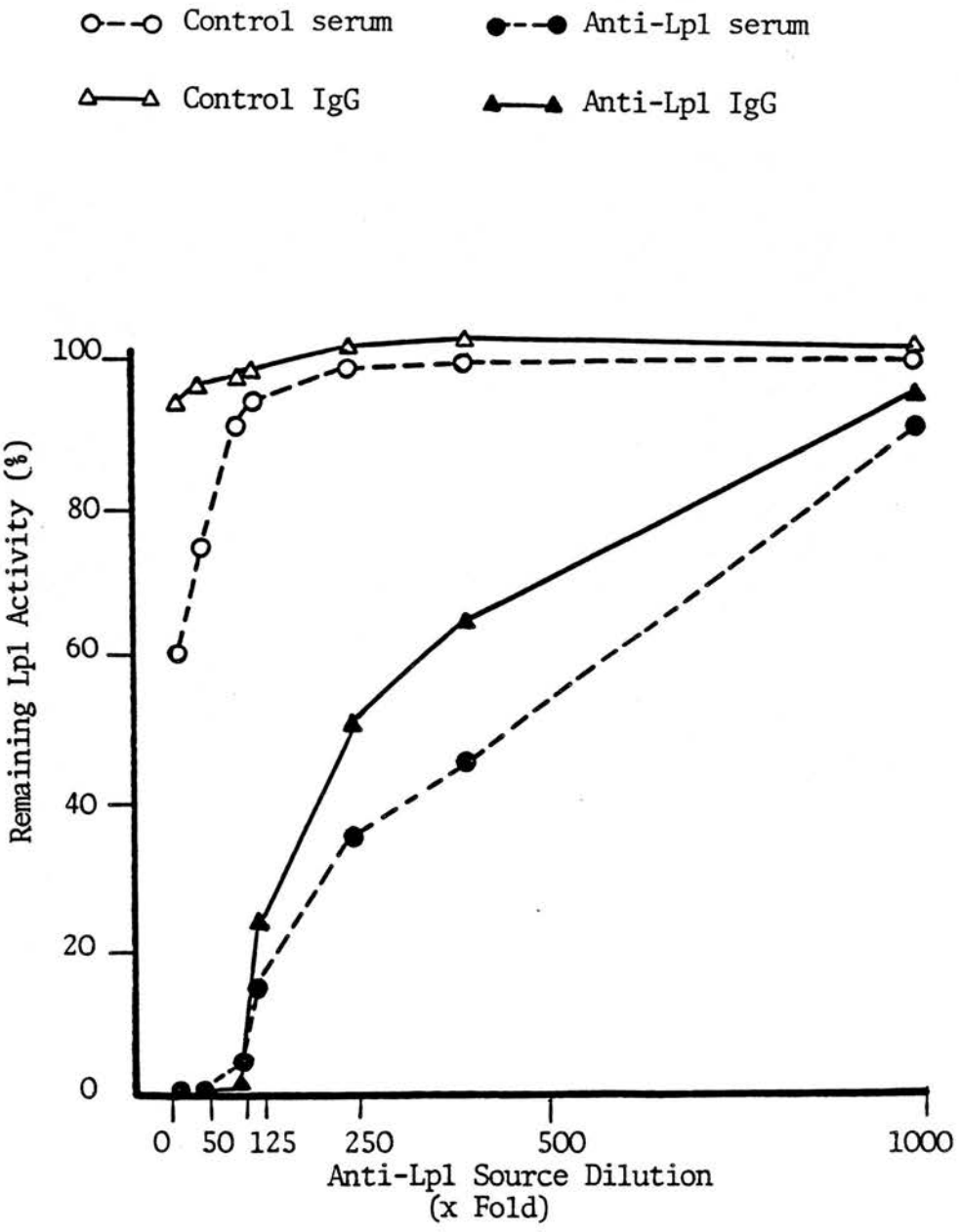
Table A.1: The in vivo potency of anti-lipoprotein lipase (anti-Lpl) antiserum and purified IgG as measured by the change in the plasma concentration of triglyceride-rich lipoproteins an hour after its intravenous administration (see Chapter 4 for practical details)

ANTIBODY SOURCE	N	LIPOPROTEIN CONCENTRATION[1]
Control Serum	3	10.9±14.4
Anti-Lpl Serum	3	481.9±221
Anti-Lpl IgG	3	1443.0±273

[1] The lipoprotein concentration is expressed as the percentage change (± SEM) over the hour

Fig A :The in vitro potency of anti-lipoprotein lipase (anti-Lpl) sources as determined by the inhibition of a fixed amount of purified adipose tissue lipoprotein lipase (Lpl)(see Chapter 2).

Serum (circles joined bybroken line) and purified immunoglobulin G (IgG) (triangles joined by solid line) from control sheep (open symbols) and from a sheep immunized against hen Lpl (closed symbols) were compared. The purified IgG sources were diluted to their original protein concentration in the serum (13mg/ml)



CHAPTER 7

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